10/585373

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MODIFIED MOLECULAR ARRAYS

FIELD OF THE INVENTION

This invention relates to the construction of arrays of
5   molecules. In particular, the invention relates to the
preparation of a hydrogel surface useful in the formation
and manipulation of arrays of molecules, particularly
polynucleotides and to the chemical modification of these
and other arrays.

10

BACKGROUND

Advances in the study of molecules have been led, in
part, by improvement in technologies used to characterise
the molecules or their biological reactions. In particular,
15   the study of nucleic acids, such as DNA and RNA, and other
large biological molecules, such as proteins, has benefited
from developing technologies used for sequence analysis and
the study of hybridisation events.

An example of the technologies that have improved the
20   study of nucleic acids is the development of fabricated
arrays of immobilised nucleic acids. These arrays typically
consist of a high-density matrix of polynucleotides
immobilised onto a solid support material. Fodor et al.,
Trends in Biotechnology (1994) 12:19-26, describe ways of
25   assembling the nucleic acid arrays using a chemically
sensitised glass surface protected by a mask, but exposed at
defined areas to allow attachment of suitably modified
nucleotides. Typically, these arrays may be described as
"many molecule" arrays, as distinct regions are formed on
30   the solid support comprising a high density of one specific
type of polynucleotide.

An alternative approach is described by Schena et al.,
Science (1995) 270:467-470, where samples of DNA are
positioned at predetermined sites on a glass microscope
35   slide by robotic micropipetting techniques.

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A further development in array technology is the attachment of the polynucleotides to a solid support material to form single molecule arrays (SMAs). Arrays of this type are disclosed in WO00/06770. The advantage of these arrays is that reactions can be monitored at the single molecule level and information on large numbers of single molecules can be collated from a single reaction.

Although these arrays offer particular advantages in sequencing experiments, the preparation of arrays at the single molecule level is more difficult than at the multi-molecule level, where losses of target polynucleotide can be tolerated due to the multiplicity of the array. Moreover, where the sequence of a polynucleotide is determined by sequential incorporations of labelled nucleotides, a further problem which arises is the occurrence of non-specific binding of nucleotides to the array, for example to the surface of the array. There is, therefore, a constant need for improvements in the preparation of arrays of molecules, particularly polynucleotides, for example single molecule arrays of polynucleotides, for sequencing procedures.

Solid-supported molecular arrays have been generated previously in a variety of ways. Indeed, the attachment of biomolecules (such as proteins and nucleic acids, e.g. DNA) to a variety of supports/substrates (e.g. silica-based substrates such as glass or plastics or metals) underpins modern microarray and biosensor technologies employed for genotyping, gene expression analysis and biological detection.

In nearly all examples where biomolecules have been immobilised on solid supports, the attachment chemistry is designed around the support. For example, silanes (e.g. functionalised silanes such as chloro- or alkoxy-silanes) are commonly used to modify glass; thiols are often used to modify the surface of gold. A potential problem here is that the agents used to modify one surface are often

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unsuitable for modifying the surface of another support.
For example, thiols cannot be used to modify glass, nor can
silanes be used to modify gold.

Silica-based substrates such as silica or glass are
often employed as supports on which molecular arrays are
constructed. It would be desirable to be able to use
chemistry useful in modifying such supports with other
supports.

Prior to the construction of any silica-based solid-
supported arrays, the support surface is generally
thoroughly cleaned. With silica-based substrates, the
resultant cleaned surface possesses hydroxyl groups which
are either neutral and/or deprotonated and thus negatively
charged. As a result there is a degree of resistance to
non-specific binding of nucleotides used in sequencing
experiments. Either the neutral hydroxyl groups do not
attract the negatively charged nucleotides, or the
deprotonated groups' negative charge serves to repel the
nucleotides. Regardless, the effect of the surface towards
the non-specific, and undesired, binding of nucleotides is
not high and it is desirable to lessen the extent of non-
specific binding in sequencing experiments. This serves to
reduce background "noise" during the detection of each
individual nucleotide in each step in sequencing
experiments.

Another way in which polynucleotides (and other
molecules) have been displayed previously on the surface of
solid support is through the use of hydrogels. Molecular
arrays, e.g. microarrays, of molecules, particularly
polynucleotides, are of use in techniques including nucleic
acid amplification and sequencing methods. In preparing
hydrogel-based solid-supported molecular arrays, a hydrogel
is formed and molecules displayed from it. These two
features - formation of the hydrogel and construction of the
array - may be effected sequentially or simultaneously.

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Where the hydrogel is formed prior to formation of the
array, it is typically produced by allowing a mixture of
comonomers to polymerise. Generally, the mixture of
comonomers contain acrylamide and one or more comonomers,
5   the latter of which permit, in part, subsequent
immobilisation of molecules of interest so as to form the
molecular array.

The comonomers used to create the hydrogel typically
contain a functionality that serves to participate in
10 crosslinking of the hydrogel and/or immobilise the hydrogel
to the solid support and facilitate association with the
target molecules of interest.

Generally, as is known in the art, polyacrylamide
hydrogels are produced as thin sheets upon polymerisation of
15 aqueous solutions of acrylamide solution. A multiply
unsaturated (polyunsaturated) crosslinking agent (such as
bisacrylamide) is generally present; the ratio of acrylamide
to bisacrylamide is generally about 19:1. Such casting
methods are well known in the art (see for example Sambrook
20 et al., 2001, Molecular Cloning, A Laboratory Manual, 3rd
Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
Laboratory Press, NY) and need not be discussed in detail
here.

As an alternative to the use of hydrogel-supported
25 molecular arrays, the use of polyelectrolyte multilayers
(PEMs) has been reported (E.P. Kartov et al., Biotechniques
(March 2003), 34:505-510; and I. Braslavsky et al., Proct
Nat. Acad. Sci (1 April 2003), 100 (7), 3960-3964) to allow
sequencing experiments to be conducted in which
30 fluorescently labelled molecules are incorporated into DNA
strands and then identified by fluorescence microscopy. The
authors report that, by using PEMs, the charge density on
the surface may be tuned so as to repel labelled nucleotides
selectively by constructing the PEMs such that the final
35 layer bears a negative charge.

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Accordingly, the authors describe such a PEM which, after its construction, was used in the formation of a molecular array. The latter was formed initially by biotinylating the surface using a commercially available kit
5   (EZ-Link™ kit from Pierce Chemical (Rockford, IL, USA)). The biotinylated PEM was then coated with Streptavidin-Plus™ (Prozyme, San Leandro, CA, USA) to which biotinylated DNA was attached. In this way the biotinylated DNA is attached to covalently bound biotin through specific non-
10   covalent interactions to "sandwiched" streptavidin molecules.

The authors of *E.P. Kartov et al.* (*infra*) and *I. Braslavsky et al.* (two authors are common to both publications) report that the final, negatively charged,
15   polyacrylic acid layer is intended to prevent negatively charged labelled nucleotides binding to the surface. It is clear, however, that this was not successful in every instance since it is reported in *I. Braslavsky et al.* (*infra*) that the identity of the third or fourth
20   incorporated nucleotide could not be determined (was "ambiguous"). According to the authors, this was caused by "increasing non-specific binding of unincorporated nucleotides".

Accordingly, there exists a need for a method of
25   providing arrays of molecules, particularly polynucleotides, which arrays have a lesser tendency to interact non-specifically with other molecules (and in particular (optionally fluorescently labelled) nucleotides used in sequencing experiments) than those available in the prior
30   art. There is also a need for a general method for modifying a solid support to allow the preparation of supports useful in the preparation of arrays.

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SUMMARY OF THE INVENTION

Heretofore, some form of covalent surface modification of the solid support has been practised in order to achieve satisfactory immobilisation of either hydrogel-based molecular arrays or hydrogels to which it is desired to array molecules. Surprisingly, we have found that such functional modification of the support is not necessary in order to achieve satisfactory immobilisation of arrays of molecules of interest, in particular polynucleotides. In order to make useful supported arrays capable of binding molecules of interest, we have found that a mixture of comonomers comprising at least one hydrophilic monomer and a functionalised comonomer (functionalised to the extent that the monomer once incorporated into the polymer is capable of binding the molecule of interest to the surface of the hydrogel) may be polymerised so as to form a hydrogel capable of being immobilised on a solid supported, preferably a silica-based, substrate.

Viewed from one aspect, therefore, the invention provides a method of preparing a hydrogel immobilised to a solid support comprising polymerising on said support a mixture of:
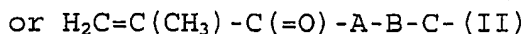
(i) a first comonomer which is acrylamide, methacrylamide, hydroxyethyl methacrylate or N-vinyl pyrrolidinone; and

(ii) a second comonomer which is a functionalised acrylamide or acrylate of formula (I):

$$H_2C=C(H)-C(=O)-A-B-C \quad (I);$$
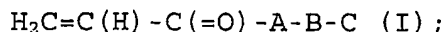
or a methacrylate or methacrylamide of formula (II):

$$or \quad H_2C=C(CH_3)-C(=O)-A-B-C- \quad (II)$$

(wherein:

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A is NR or O, wherein R is hydrogen or an optionally substituted saturated hydrocarbyl group comprising 1 to 5 carbon atoms;

-B- is an optionally substituted alkylene biradical of formula $-(CH_2)_n-$ wherein n is an integer from 1 to 50; and wherein n = 2 or more, one or more optionally substituted ethylene biradicals $-CH_2CH_2-$ of said alkylene biradical may be independently replaced by ethenylene and ethynylene moieties; and wherein n=1 or more, one or more methylene biradicals $-CH_2-$ may be replaced independently with an optionally substituted mono- or polycyclic hydrocarbon biradical comprising from 4 to 50 carbon atoms, or a corresponding heteromonocyclic or heteropolycyclic biradical wherein at least 1 CH_2 or CH_2 is substituted by an oxygen sulfur or nitrogen atom or an NH group; and

C is a group for reaction with a compound to bind said compound covalently to said hydrogel) to form a polymerised product,

characterised in that said method is conducted on, and immobilises the polymerised product to, said support which is not covalently surface-modified.

Viewed from a second aspect, the invention provides a solid-supported hydrogel obtainable according to the method of the first invention.

Viewed from a third aspect, the invention provides a method of preparing a solid-supported hydrogel-based molecular array by attaching one or more molecules of interest to reactive sites present in the solid-supported hydrogel according to the invention.

In a particular embodiment the invention provides a method of preparing a solid-supported hydrogel-based molecular array which is a clustered array by attaching oligonucleotide primers to reactive sites present in the solid-supported hydrogel and performing nucleic acid amplification of a template using the bound primers.

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Viewed from a fourth aspect, the invention provides a solid-supported hydrogel-based molecular array obtainable according to the third aspect of the invention.
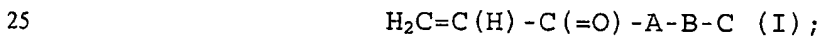
Viewed from a fifth aspect, the invention provides the
5   use of a molecular array according to the fourth aspect of the invention in any method of analysis which requires interrogation of the molecules of interest or molecules bound thereto.

The use of solid-supported hydrogel arrays in single
10   molecule array applications has not been conducted previously. Thus viewed from a sixth aspect, the invention provides the use of solid-supported hydrogel arrays in single molecule array applications, preferably wherein said arrays are obtainable, and generally obtained, by a method
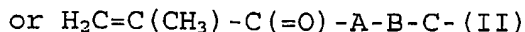15   comprising:

(1)   preparing a hydrogel immobilised to a solid support comprising polymerising on said support a mixture of:

(i)   a first comonomer which is acrylamide,
20   methacrylamide, hydroxyethyl methacrylate or N-vinyl pyrrolidinone; and

(ii)   a second comonomer which is a functionalised acrylamide or acrylate of formula (I):

25                       $H_2C=C(H)-C(=O)-A-B-C$ (I);

or a methacrylate or methacrylamide of formula (II):

                          or $H_2C=C(CH_3)-C(=O)-A-B-C-$ (II)
30   (wherein:

A is NR or O, wherein R is hydrogen or an optionally substituted saturated hydrocarbyl group comprising 1 to 5 carbon atoms;

-B- is an optionally substituted alkylene biradical of
35   formula $-(CH_2)_n-$ wherein n is an integer from 1 to 50; and

wherein n = 2 or more, one or more optionally substituted ethylene biradicals -CH_2CH_2- of said alkylene biradical may be independently replaced by ethenylene and ethynylene moieties; and wherein n=1 or more, one or more methylene

5   biradicals -CH_2- may be replaced independently with an optionally substituted mono- or polycyclic hydrocarbon biradical comprising from 4 to 50 carbon atoms, or a corresponding heteromonocyclic or heteropolycyclic biradical wherein at least 1 CH_2 or CH_2 is substituted by an oxygen

10   sulfur or nitrogen atom or an NH group; and

C is a group for reaction with a compound to bind said compound covalently to said hydrogel) to form a polymerised product, and

(2)   attaching one or more molecules of interest to

15   reactive sites present in the hydrogel produced in step (1).

The invention also provides the use of, and methods of using, arrays, preferably single molecule arrays according to invention, in the interrogation of the molecules in said array.

20   One of the advantages of the hydrogel-based molecular arrays and hydrogels of the invention, it has been found, is that omission of a covalent surface-modification step (particularly of the solid support) affords a surface having greater passivity than in the prior art, particularly when

25   compared to those instances where the use of the silane-modifying agents described above with silica-based substrates are employed.

The provision of a surface which leads to as little as possible aspecific surface contamination is clearly

30   advantageous where the hydrogels are used to construct arrays, such as microarrays, and preferably clustered arrays or SMAs, to be used in sequencing reactions. Notwithstanding this, however, the hydrogels of this invention have functionality used in forming, or reacting

35   with, the molecules which are arrayed. Consequentially,

these hydrogels suffer too, albeit to a more limited extent than prior art hydrogels supported upon a functionally modified support, from a degree of aspecific nucleotide binding during sequencing.

Surprisingly, we have found that the solid-supported hydrogel-based molecular arrays of the invention may be still further improved by effecting certain modifications to these arrays after their formation but before initiation of any manipulation of, e.g. interrogation of, the molecules in the array. These arrays are of even greater advantageousness in, for example, polynucleotide sequencing reactions because the surfaces of the arrays may be rendered more passive, and thus less reactive, towards molecules such as optionally labelled nucleotides.

Accordingly the method according to the third aspect of the invention preferably contains the additional step of applying to the array so produced polyelectrolyte or neutral polymers. This improvement is of corresponding benefit to the other aspects of the invention directed to the arrays themselves, and the uses, and methods of using, such arrays.

The improvement to the solid-supported hydrogel-based molecular arrays, and uses of the arrays, of the invention is of general utility in the preparation and use of molecular arrays. It will be appreciated from the foregoing discussion that, in the preparation of arrays of molecules to date, particularly in the preparation of arrays of polynucleotides, these have invariably been assembled by initial preparation of the support, whether this be achieved by modification of a silica-based substrate, or formation of a PEM on a glass substrate, or formation of a hydrogel on glass or other solid supports. Only once the constitution of the solid support has been finalised is the array formed by reaction between the support and the molecules of interest. The array is then used, without further modification, in methods of analysis such as sequencing

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experiments whereby the molecules, typically polynucleotides, are interrogated.

Viewed from a still further aspect, therefore, the invention provides a method of modifying a molecular array, which molecular array comprises a plurality of molecules of interest, preferably biomolecules, immobilised to a surface of a support, said method comprising the step of applying to the array polyelectrolyte or neutral polymers.

Viewed from a still further aspect, the invention provides a molecular array obtainable according to the immediately preceding aspect of the invention.

Viewed from a still further aspect, the invention provides the use of a molecular array according to the immediately preceding aspect of the invention in any method of analysis which requires interrogation of the immobilised biomolecules, or of molecules bound thereto.

The present invention will now be further described. In the following passages different aspects of the invention are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Figure 1 shows images which show the detection of fluorescence from oligonucleotides immobilised on substrates both according to and not according to the invention, in accordance with Example 11. The improved binding of phosphorothioate-terminated DNA (PS-DNA) over hydroxyl-terminated DNA may be seen;

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Fig. 2: Figure 2 shows the relative levels of positive
signal due to PS-DNA binding versus negative noise from OH-
DNA binding resultant from coupling of 1 μM PS-DNA and 1 μM
OH-DNA to plastic and fused silica (SPECTRASIL® glass)

5   detected on the surfaces whose preparation is described in
Example 10;

Fig. 3: Figure 3 shows the apparent stability of the
specifically adsorbed PS-DNA (in 50 mM phosphate buffer

10   (pH7, 65°C)) for various plastics materials is approximately
the same as that for SPECTRASIL™ glass;

Fig. 4: Figure 4 shows images of the detection of
fluorescence from oligonucleotides immobilised on substrates

15   both according to and not according to the invention, in
accordance with Example 13. The improved binding of
phosphorothioate-terminated DNA (PS-DNA) over hydroxyl-
terminated DNA may be seen.

20   Fig. 5: Figure 5 is a schematic illustration of a prior
art method of solid-phase amplification in which a mixture
of oligonucleotide primers and template strands are
simultaneously grafted onto a solid support.

25   Fig. 6: Figure 6 is a schematic illustration of a
method of solid-phase amplification according to the
invention in which oligonucleotide primers are first grafted
onto a solid support and then hybridised to template
strands.

30
Fig. 7: Figure 7 illustrates the use of spacer
nucleotides to improve efficiency of hybridisation between
an immobilised polynucleotide primer and a labelled target,
according to Example 14.

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Fig. 8: Figure 8 shows the results of hybridisation experiments carried out in microtiter plates between various concentrations of immobilised primers - with and without spacer nucleotides - and target oligonucleotides labelled with Texas red (TXR). In Figure 8(a) the identity of the immobilised primer is indicated along the top of the plate. Templates were added in groups of four wells, as shown in the key. Hybridisation was carried out in two different buffers - a PCR buffer (PCR) and 5xSSC (SSC). Each immobilised primer was tested for hybridisation with a complementary primer (i.e. P5' is complementary to P5) and with a control non-complementary primer. Figure 8(b) illustrates typical results in graphical form.

Fig. 9: Figure 9 illustrates experimental set-up for hybridisation experiments described in Example 14.

Fig. 10: Figure 10 illustrates experimental set-up and results of hybridisation experiments using various immobilised oligonucleotide primers, with and without spacer nucleotides, and complementary labelled target oligonucleotides.

Fig. 11: Figure 11 graphically represents the results of typical hybridisation experiments according to Figure 13 and Example 14.

Fig. 12: Figure 12 illustrates the results of hybridisation experiments using immobilised oligonucleotide primers containing varying numbers of spacer nucleotides and complementary labelled target oligonucleotides. Figure 12(a) shows experimental set-up and results. Figure 12(b) provides a graphical representation of the results of typical hybridisation experiments.

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DETAILED DESCRIPTION OF THE INVENTION

The invention, as described and claimed herein,
provides an improved method for displaying molecules of
5 interest, and particularly biomolecules (biological
molecules) such as polynucleotides and proteins (preferably
polynucleotides) displayed on the surface of a solid
support, preferably a solid-supported hydrogel.

The solid upon which the hydrogel is supported is not
10 limited to a particular matrix or substrate. Indeed, this
is one of the advantages of the invention: the same
chemistry used to modify silica-based substrates can be
applied to other solid supports and allows the solid support
to be adapted to suit any particular application to which it
15 is desired to be put rather than being constrained by the
surface chemistry it is possible to perform on any given
support. Solids which may be of use in the practise of this
invention thus include silica-based substrates, such as
glass, fused silica and other silica-containing materials;
20 they may also be silicone hydrides or plastic materials such
as polyethylene, polystyrene, poly(vinyl chloride),
polypropylene, nylons, polyesters, polycarbonates and
poly(methyl methacrylate). Preferred plastics material are
poly(methyl methacrylate), polystyrene and cyclic olefin
25 polymer substrates. Alternatively, other solid supports may
be used such as gold, titanium dioxide, or silicon supports.
The foregoing lists are intended to be illustrative of, but
not limited to, the invention. Preferably, the support is a
silica-based material or plastics material such as discussed
30 herein.

Advantages in using plastics-based substrates in the
preparation and use of molecular arrays include cost: the
preparation of appropriate plastics-based substrates by, for
example injection-moulding, is generally cheaper than the
35 preparation, e.g. by etching and bonding, of silica-based

substrates. Another advantage is the nearly limitless
variety of plastics allowing fine-tuning of the optical
properties of the support to suit the application for which
it is intended or to which it may be put.

5   Where metals are used as substrates, this may be
because of the desired application: the conductivity of
metals can allow modulation of the electric field in DNA-
based sensors. In this way, DNA mismatch discrimination may
be enhanced, the orientation of immobilised oligonucleotide
10   molecules can be affected, or DNA kinetics can be
accelerated.

       Preferably the support is silica-based but the shape of
the support employed may be varied in accordance with the
application for which the invention is practiced.
15   Generally, however, slides of support material, such as
silica, e.g. fused silica, are of particular utility in the
preparation and subsequent interrogration of molecules. Of
particular use in the practice of the invention are fused
silica slides sold under the trade name SPECTRASIL™. This
20   notwithstanding, it will be evident to the skilled person
that the invention is equally applicable to other
presentations of solid support (including silica-based
supports), such as beads, rods and the like.

       The genesis of the invention is the recognition by the
25   inventors that the surface of the support need not be
covalently modified in order for a hydrogel to be
immobilised thereto. As described herein, the step of
covalent surface modification may be omitted when the
comonomer mixture described and claimed herein is used to
30   produce a hydrogel.

       If it is desired to display molecules of interest, e.g.
biomolecules, these may be any biological molecule which it
is desired to analyse. Of particular interest are
polypeptides or proteins (including enzymes) and

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polynucleotides, with polynucleotides being particularly
preferred.

As used herein, the term "polynucleotide" refers to
nucleic acids in general, including DNA (e.g. genomic DNA
5   cDNA), RNA (e.g. mRNA), synthetic oligonucleotides and
synthetic nucleic acid analogs. Polynucleotides may include
natural or non-natural bases, or combinations thereof and
natural or non-natural backbone linkages, e.g.
phosphorothioates, PNA or 2'-O-methyl-RNA, or combinations
10   thereof.

Whilst it will be appreciated that the solid-supported
hydrogels of the invention are useful for the presentation
of many different types of molecules, the hydrogels are of
particular use in the formation of arrays of polynucleotides
15   and their subsequent analysis. For this reason, the
majority of the subsequent discussion will focus upon the
utility of the supported hydrogels of the invention in the
preparation of polynucleotide arrays (both single molecule
arrays and microarrays, such as clustered arrays formed by
20   nucleic acid amplification) although it is to be understood
that such applications in no way limit the invention.
Moreover, since silica-based supports are typically used to
support hydrogels and hydrogel arrays, the subsequent
discussion will focus on the use of silica-based supports.
25   Again, this is not to be considered as a limitation of the
invention; rather this is demonstrative of a particular
advantage of the invention for improving procedures directed
to constructing arrays of molecules on silica-supported
hydrogels. The improvement offered will be evident from a
30   review of the prior art.

WO00/31148 discloses polyacrylamide hydrogels and
polyacrylamide hydrogel-based arrays in which a so-called
polyacrylamide prepolymer is formed, preferably from
acrylamide and an acrylic acid or an acrylic acid derivative
35   containing a vinyl group. Crosslinking of the prepolymer

may then be effected. The hydrogels so produced are solid-supported, preferably on glass. Functionalisation of the solid-supported hydrogel may also be effected.

WO01/01143 describes technology similar to WO00/31148 but differing in that the hydrogel bears functionality capable of participating in a [2+2] photocycloaddition reaction with a biomolecule so as to form immobilised arrays of such biomolecules. Dimethylmaleimide (DMI) is a particularly preferred functionality. The use of [2+2] photocycloaddition reactions, in the context of polyacrylamide-based microarray technology is also described in WO02/12566 and WO03/014392.

US Patent No. 6,465,178 discloses the use of reagent compositions in providing activated slides for use in preparing microarrays of nucleic acids; the reagent compositions include acrylamide copolymers. The activated slides are stated to be particularly well suited to replace conventional (e.g. silylated) glass slides in the preparation of microarrays.

WO00/53812 discloses the preparation of polyacrylamide-based hydrogel arrays of DNA and the use of these arrays in replica amplification.

None of the prior art described herein discloses the preparation of a solid-supported hydrogel wherein the solid support is not covalently modified.

Once hydrogels have been formed, molecules may then be attached to them so as to produce molecular arrays, if desired. Attachment has been effected in different ways in the prior art. For example, US Patent No. 6,372,813 teaches immobilisation of polynucleotides bearing dimethylmaleimide groups to the hydrogels produced which bear dimethylmaleimide groups by conducting a [2+2] photocycloaddition step between two dimethylmaleimide groups - one attached to the polynucleotide to be immobilised and one pendant from the hydrogel.

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Where the molecular array is formed after generation of
the hydrogel, two strategies have been employed to achieve
this end. Firstly, the hydrogel may be modified chemically
after it is produced. Problems with this approach include
5   an overall low efficiency in the preparation of the array
and the low stability relating to the attachment chemistry,
particularly upon exposure to high temperatures, ionic
solutions and multiple wash steps.

A more common alternative is to effect polymerisation
10   with a comonomer having a functionality primed or pre-
activated to react with the molecules to be arrayed.

Alternatives to initial formation of hydrogels followed
by subsequent arraying of molecules thereto have been
described in the prior art where the array is formed at the
15   same time as the hydrogel is produced. This may be effected
by, for example, direct copolymerisation of acrylamide-
derivatized polynucleotides. An example of this approach is
described in WO01/62982 in which acrylamide-derivatized
polynucleotides are mixed with solutions of acrylamide and
20   polymerisation is effected directly.

Mosaic Technologies (Boston, Massachusetts, USA)
produce ACRYDITE™ (an acrylamide phosphoramidite) which can
be reacted with polynucleotides prior to copolymerisation of
the resultant monomer with acrylamide.

25   Efimov et al. (Nucleic Acids Research, 1999, 27 (22),
4416-4426) disclose a further example of a simultaneous
formation of hydrogel/array in which copolymerisation of
acrylamide, reactive acrylic acid derivatives and the
modified polynucleotides having 5'- or 3'- terminal
30   acrylamide groups is effected.

The above-described techniques, however, in which the
hydrogel is generated simultaneously with the array by
introduction of appropriate comonomers bearing the molecules
of interest, suffer from problems including damage to the
35   molecules of interest during polymerisation.

A variety of solid supports have been used in the prior
art to generate hydrogel-based solid-supported molecular
arrays. These include those supports discussed earlier.
Generally the preferred solid support comprises a silica-
based substrate. Examples of silica-based substrates
include fused silica and glass.

To the best of our knowledge, in all instances of
silica-based supported hydrogels described in the prior art
the silica is chemically modified in some way so as to
attach covalently a chemically reactive group capable of
reacting with either the hydrogel or a partially formed
hydrogel (e.g. a prepolymer (PRP)). The surface-activating
agent is typically an organosilicon (organosilane) compound.
Most commonly, it is γ-methacryloxypropyltrimethoxysilane,
known as "Bind Silane" or "Crosslink Silane" and
commercially available from Pharmacia, although other
silicon-based surface-activating agents are also known, such
as monoethoxydimethylsilylbutanal, 3-mercaptopropyl-
trimethoxysilane and 3-aminopropyltrimethoxysilane (all
available from Aldrich). In this way, pendant functional
groups such as amine groups, sulfhydryl groups, aldehydo
groups or polymerisable groups (e.g. olefins) may be
attached to the silica.

It will be clear from the preceding discussion that the
invention is particularly useful when the solid support used
is silica-based since the silca-based support need not be
covalently modified by its preactivation with a silylating
agent as described above in order to immobilise the hydrogel
thereto. Clearly, however, the polymers described herein
may still be (in certain aspects of the invention) attached
to silica-based supports which have been surface-activated,
e.g. with an organosilane molecule as described above; the
full advantages of the invention will, however, not be
obtained in this way. They may also be, of course, attached
to the other solid supports disclosed herein, in particular

plastics such as poly(methyl methacrylate) and polyolefins.
Such plastics are readily available commercially, e.g. from
Arnic, Corning, Zeon Chemical Ltd and others.

5   The surface-modification of silica-based solid supports
by means other than covalent attachment of an organosilicon
moiety is not excluded from the scope of this invention.
Preferably, however, no activation of the silica - by
covalent modification of a surface thereof or by any other
means - is effected prior to effecting polymerisation
10   thereon.

It will be understood that the terms such as "covalent
surface-modification", "covalent surface-modifying" and
"covalent surface-modified" do not embrace the simple
cleaning and/or drying of substrates, particularly silica
15   substrates, prior to their use. Generally such steps will
be conducted prior to any polymerisation but do not
constitute a surface-modifying step since no covalent
modification of a surface is essentially effected by such
steps.

20   Where the substrate is silica-based, cleaning will be
achieved by contact with one or more organic solvents such
as acetone, isopropanol (IPA), ethanol and the like, or with
water or aqueous acidic or alkaline solutions such as dilute
hydrochloric or sulphuric acids or dilute sodium hydroxide.
25   Silica-based supports may be also cleaned by contact with a
detergent solution (e.g. Decon 90). These various cleaning
steps may be conducted individually or in combination, e.g.
sequentially. Drying may be effected, for example, by
heating of silica slides at temperatures of from 40°C to
30   200°C, preferably 80°C to 150°C for between 5 minutes to 24
hours, preferably at temperatures of around 120°C, and
preferably for 1 to 2 hours.

The cleaning and drying steps described above will be
and are understood by those skilled in the art not to
35   constitute any form of covalent surface modification (and in

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particular no form of covalent surface modification in which
an organosilicon (organosilane) moiety is attached). Such
steps serve to effect removal of surface contamination (e.g.
dirt or dust) and will generally be conducted prior to use
5   of silica-based materials in most scientific applications.
Heating of glass to very high temperatures (e.g. 1000°C or
higher or even 300°C or higher) or by contact with materials
known to dissolve, or etch glass (such as hydrofluoric
acid), whilst unlikely to be conducted in the cleaning of
10   silica-based substrates in advance of practice of this
invention, is not to be considered as constituting a form of
surface modification.

        For substrates that are not silica-based other cleaning
techniques may be appropriate, as will be apparent to those
15   skilled in the art. For example, plastic substrates may be
cleaned by contact with (e.g. immersion in) any convenient
detergent (Decon 90 is an example) followed by thorough
rinsing with water, preferably purified water such as
MilliQ, prior to drying.

20   The methods by which the mixture of comonomers are
polymerised in the invention are not characteristic of this
invention and will be known to the skilled person (e.g. by
recourse to Sambrook et al. (supra). Generally, however,
the polymerisation will be conducted in an aqueous medium,
25   and polymerisation initiated by any suitable initiator.
Potassium or ammonium persulfate as an initiator is
typically employed. Tetramethylethylenediamine (TMEDA or
TEMED) may be and generally is used to accelerate the
polymerisation.

30   It is important to note that, in contrast to the
teaching of hydrogel preparation in the prior art concerned
with the preparation of molecular arrays, it is not
necessary that a polyunsaturated crosslinking agent such as
bisacrylamide or pentaerythritol tetraacrylate is present in
35   the mixture which is polymerised; nor is it necessary to

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form PRP-type intermediates and crosslink them. It is one
of the surprising features of the invention that
satisfactory stability of the immobilised array may be
achieved in the absence of such crosslinking agents or PRP
5   crosslinking steps. The absence of a polyunsaturated
crosslinking agent (such as *bis*acrylamide or pentaerythritol
tetraacrylate) is a preferred feature in all aspects of this
invention directed toward hydrogels, or uses or methods of
the preparation thereof. Thus it is a preferred feature of
10   such aspects of the invention that the mixture to be
polymerised does not comprise such a polyunsaturated
crosslinking agent and that the monomers to be polymerised
consist essentially of those defined in claim 1 (i.e. no
polyunsaturated crosslinking monomer is included in the
15   mixture).

        Generally, in producing hydrogels according to this
invention, only one compound of formulae (I) or (II) will be
used.

        We have found that use of a compound of the formulae
20   (I) or (II) permits formation of a hydrogel capable of being
immobilised to solid supports, preferably silica-based solid
supports. The compounds of these formulae comprise portions
A, B and C as defined herein.

        Biradical A may be oxygen or N(R) wherein R is hydrogen
25   or a C_{1-5} alkyl group. Preferably, R is hydrogen or methyl,
particularly hydrogen. Where R is a C_{1-5} alkyl group, this
may contain one or more, e.g. one to three substituents.
Preferably, however, the alkyl group is unsubstituted.

        Biradical B is a predominantly hydrophobic linking
30   moiety, connecting A to C and may be an alkylene biradical
of formula $-(CH_2)_n-$, wherein n is from 1 to 50. Preferably
n is 2 or more, e.g. 3 or more. Preferably n is 2 to 25,
particularly 2 to 15, more particularly 4 to 12, for example
5 to 10.

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Where n in $-(CH_2)_n-$ is 2 or more, one or more
biradicals $-CH_2CH_2-$ (-ethylene-) may be replaced with
ethenylene or ethynylene biradicals. Preferably, however,
the biradical B does not contain such unsaturation.

Additionally, or alternatively, where n in $-(CH_2)_n-$ is
1 or more, one or more methylene radicals $-CH_2-$ in B may be
replaced with a mono- or polycyclic biradical which
preferably comprises 5 to 10 carbon atoms e.g. 5 or 6 carbon
atoms. Such cyclic biradicals may be, for example, 1,4-,
1,3- or 1,2-cyclohexyl biradicals. Bicylic radicals such as
napthyl or decahydronaphthyl may also be employed.
Corresponding heteroatom-substituted cyclic biradicals to
those homocyclic biradicals may also be employed, for
example pyridyl, piperidinyl, quinolinyl and
decahydroquinolinyl.

It will be appreciated that the scope of -B- is thus
not particularly restricted. Most preferably, however, -B-
is a simple, unsubstituted, unsaturated alkylene biradical
such as a C_3-C_{10} alkylene group, optimally C_5-C_8, such as n-
pentylene: $-(CH_2)_5-$.

Where an alkyl group (or alkylene, alkenylene etc) is
indicated as being (optionally) substituted, substituents
may be selected from the group comprising hydroxyl, halo
(i.e. bromo, chloro, fluoro or iodo), carboxyl, aldehydo,
amine and the like. The biradical -B- is preferably
unsubstituted or substituted by fewer than 10, preferably
fewer than 5, e.g. by 1, 2 or 3 such substituents.

Group C serves to permit attachment of molecules of
interest after formation of the hydrogel. The nature of
Group C is thus essentially unlimited provided that it
contains a functionality allowing reaction between the
hydrogel and molecules of interest. Preferably, such a
functionality will not require modification prior to
reaction with the molecule of interest and thus the C group
is ready for direct reaction upon formation of the hydrogel.

Preferably such a functionality is a hydroxyl, thiol, amine, acid (e.g. carboxylic acid), ester and haloacetamido, haloacetamido and in particular bromoacetamido being particularly preferred. Other appropriate C groups will be evident to those skilled in the art and include groups comprising a single carbon-carbon double bond which is either terminal (i.e. where a C group has an extremity terminating in a carbon-carbon double bond) or where the carbon-carbon double bond is not at a terminal extremity. When a C group comprises a carbon-carbon double bond, this is preferably fully substituted with C_{1-5} alkyl groups, preferably methyl or ethyl groups, so that neither carbon atom of the C=C moiety bears a hydrogen atom.

The C moiety may thus comprise, for example, a dimethylmaleimide moiety as disclosed in US 6,372,813, WO01/01143, WO02/12566 and WO03/014394.

The (meth)acrylamide or (meth)acrylate of formula (I) or (II) which is copolymerised with acrylamide, methacrylamide, hydroxyethyl methacrylate or N-vinyl pyrrolidinone is preferably an acrylamide or acrylate, i.e. of formula (I). More preferably it is an acrylamide and still more preferably it is an acrylamide in which A is NH.

The reaction between a comonomer of formula (I) or (II) and acrylamide, methacrylamide, hydroxyethyl methacrylate or N-vinyl pyrrolidinone methacrylamide, particularly acrylamide, has been found to afford hydrogels particularly suitable for use in the generation of molecular arrays. However, it will be appreciated by those skilled in the art that analogous copolymers may be formed by the reaction between comonomers of formula (I) or (II) and any vinylogous comonomer, hydroxyethylmethacrylate and n-vinyl pyrrolidinone being two examples of such vinylogous comonomers.

Control of the proportion of monomer of formula (I) or (II) to that of the first comonomer (e.g. acrylamide and/or

methacrylamide, preferably acrylamide) allows adjustment of
the physical properties of the hydrogel obtained on
polymerisation. It is preferred that the comonomer of
formula (I) or (II) is present in an amount of ≥1 mol%,
preferably ≥2 mol% (relative to the total molar quantity of
comonomers) in order for the hydrogel to have optimum
thermal and chemical stability under conditions typically
encountered during the preparation, and subsequent
manipulation, of the molecular arrays produced from the
hydrogels. Preferably, the amount of comonomer of formula
(I) or (II) is less than or equal to about 5 mol%, more
preferably less than or equal to about 4 mol%. Typical
amounts of comonomer of formula (I) or (II) used are 1.5-3.5
mol%, exemplified herein by about 2% and about 3%.

The amounts of acrylamide or methacrylamide from which
the hydrogels are primarily constructed are those typically
used to form hydrogels, e.g. about 1 to about 10% w/v,
preferably less than 5 or 6% w/v, e.g. about 1 to about 2%
w/v. Again, of course, the precise nature of the hydrogel
may be adjusted by, in part, control of the amount of
acrylamide or methacrylamide used.

When forming the hydrogels, acrylamide or
methacrylamide may be dissolved in water and mixed with a
solution of a comonomer of formula (I) or (II). The latter
may be conveniently dissolved in a water-miscible solvent,
such as dimethylformamide (DMF), or water itself. The most
appropriate solvent may be selected by the skilled person
and shall depend upon the structure of the comonomer of
formula (I) or (II).

The methods by which the monomers of formula (I) or
(II) are synthesised will be evident to those skilled in the
art. By way of example, the synthesis of a particulary
preferred monomer (of formula (I) wherein A = NH, -B- =
-(CH$_2$)$_5$- and -C = -N(H)-C(=O)CH$_2$Br is provided as an example
hereinafter.

As noted above, the general methods by which the
polymerisation is carried out are known to those skilled in
the art. For example, generally acrylamide or
methacrylamide is dissolved in purified water (e.g. Milli Q)
5   and potassium or ammonium persulfate dissolved separately in
purified water. The comonomer of formula (I) or (II) may be
conveniently dissolved in a water-miscible organic solvent,
e.g. glycerol, ethanol, methanol, dimethylformamide (DMF)
etc. TEMED may be added as appropriate. Once formulated (a
10 typical preparation is described in the examples), the
mixture is polymerised with as little delay as possible
after its formulation.

The polymerisation process may be conducted by any
convenient means. Several examples are described in the
15 experimental section below.

The hydrogels according to this invention are of
particular utility in the preparation of arrays of
molecules, particularly single molecule arrays (SMAs) or
clustered arrays and in particular SMAs or clustered arrays
20 of polynucleotides.

It is noted above that it is a surprising feature of
relevant aspects this invention that it is possible to omit
the inclusion of a polyunsaturated crosslinking agent.
Where such a crosslinking agent is omitted, as is
25 preferable, it is possible to make thinner hydrogels than
have been achievable heretofore. In particular, omission of
such crosslinking agents allows preparation of hydrogels
having thicknesses of less than about 100 nm, for example
less than 75 nm; the hydrogels may be less than about 50 nm
30 thick.

Such hydrogels are of particular use where they are
used to generate arrays, in particular single molecule
arrays or clustered arrays, particularly of nucleotides, and
in the interrogation of such arrays wherein fluorescently
35 labelled nucleotides are incorporated into a nascent

polynucleotide and then detected. Such techniques are
described in greater detail hereinafter.

There are a number of advantages of the hydrogels
produced according to this invention. With particular

5   regard to silica-based substrates, the invention allows the
avoidance of the covalent chemical modification (especially
with silicon-containing agents) of the silica-based
substrate in order for the hydrogels produced on the support
be immobilised thereto. Thus, the invention permits

10 immobilisation of hydrogels on a variety of solid supports
upon which such immobilisation has not been reported
previously.

By "immobilising" of a hydrogel on a support is meant
that the supported hydrogels are associated with the support

15 in such a way so as to remain as a layer upon the support
under conditions encountered during preparing and
manipulating (e.g. interrogating) molecular arrays. Such
preparations and manipulations are described in greater
detail hereinafter and are known to those skilled in the

20 art.

A further advantage of the supported hydrogels of the
invention is avoidance of the need for chemical modification
of the hydrogel (i.e. post polymerisation) in order to
attach of molecules of interest. With the "C" groups

25 described herein, appropriately functionalised molecules may
be attached directly to the hydrogel.

A further advantage is the passivity, i.e. the
essential lack of reactivity, of the surface of the hydrogel
towards non-specific adherence of molecules (e.g.

30 fluorescently labelled nucleotides) to the surface; such
adherents would otherwise create unwanted "noise" during
subsequent manipulation of molecular arrays formed from the
hydrogels, particularly in the manipulation of SMAs or
clustered arrays.

As noted above, it has been found that omission of a covalent surface-modification step results in a surface having greater passivity than in the prior art, particularly when compared to those instances where the use of the
5   silane-modifying agents described above with silica-based substrates are employed.

The provision of a surface which leads to as little as possible aspecific surface contamination is clearly advantageous where the hydrogels are used to construct
10   arrays to be used in SMA applications. Of course, where the hydrogels are used to construct clustered microarrays, minimising surface contamination to the greatest extent possible is also advantageous.

In accordance with preferred aspects of the invention,
15   the solid-supported hydrogel-based molecular arrays of the invention may be treated with polyelectrolyte or neutral polymers to afford arrays, preferably SMAs or clustered arrays, particularly SMAs or clustered arrays of polynucleotides, the surfaces of which have enhanced
20   passivity towards, for example, aspecific binding with nucleotides (e.g. labelled nucleotides) used in sequencing and other interrogative methods of using the arrays. Also, the invention, as described and claimed herein, provides an improved method by which molecules of interest (preferably
25   biomolecules such as those identified above), preferably polynucleotides and proteins (especially preferably polynucleotides) may be displayed by modifying existing, i.e. preprepared, arrays of molecules. It will be appreciated that, since this aspect of the invention lies in
30   the modification of existing molecular arrays, the nature of the molecular array treated according to the method of the invention is not of any particular importance. This notwithstanding, it is preferred that the arrays treated are in accordance with the fourth aspect of this invention.
35   Likewise, the nature of the biomolecules arrayed, or the

means by which they are arrayed, is of lesser importance
than the requirement for the modification step whereby the
array is treated with polyelectrolyte or neutral polymers.

On account of the particular utility of molecular
arrays, preferably SMAs or clustered arrays, most preferably
SMAs or clustered arrays of polynucleotides, in sequence
determination methods, the preceding discussion has
focussed, and subsequent discussion shall focus, on this
utility of the invention although it is to be understood
that the invention is not to be considered to be so limited.

Similarly, the make-up of the solid support of the
array to be modified, or the type of the array to be
modified is not of as great importance as the manner in
which it is treated (modified) according to the invention.
SMAs and clustered arrays, however, and preferably SMAs and
clustered arrays of polynucleotides are particularly
advantageous.

The term "single molecule array" or "SMA" as used
herein refers to a population of polynucleotide molecules,
distributed (or arrayed) over a solid support, wherein the
spacing of any individual polynucleotide from all others of
the population is such that it is possible to effect
individual resolution, or interrogation, of the
polynucleotides. The target nucleic acid molecules
immobilised onto the surface of the solid support should
thus be capable of being resolved by optical means. This
means that, within the resolvable area of the particular
imaging device used, there must be one or more distinct
signals, each representing one polynucleotide. This may be
achieved, preferably wherein the spacing between adjacent
polynucleotide molecules on the array is at least 100 nm,
more preferably at least 250 nm, still more preferably at
least 300 nm, even more preferably at least 350 nm. Thus,
each molecule is individually resolvable and detectable as a
single molecule fluorescent point, and fluorescence from

said single molecule fluorescent point also exhibits single step photobleaching.

Clusters of substantially identical molecules do not exhibit single point photobleaching under standard operating conditions used to detect/analyze molecules on arrays. The intensity of a single molecule fluorescence spot is constant for an anticipated period of time after which it disappears in a single step. In contrast, the intensity of a fluorescence spot comprised of two or more molecules, for example, disappears in two or more distinct and observable steps, as appropriate. The intensity of a fluorescence spot arising from a cluster consisting of thousands of similar molecules, such as those present on the arrays consisting of thousands of similar molecules at any given point, for example, would disappear in a pattern consistent with an exponential decay. The exponential decay pattern reflects the progressive loss of fluorescence by molecules present in the cluster and reveals that, over time, fewer and fewer molecules in the spot retain their fluorescence.

The term "clustered array" refers to an array wherein distinct regions or sites on the array comprise multiple polynucleotide molecules that are not individually resolvable by optical means. Depending on how the array is formed each region or site on the array may comprise multiple copies of one individual polynucleotide molecule or even multiple copies of a small number of different polynucleotide molecules (e.g. multiple copies of two complementary nucleic acid strands). In a preferred embodiment the term "clustered array" refers to an array produced by solid-phase amplification of a target or template polynucleotide, wherein amplified copies of the target or template become covalently bound to the solid support during amplification.

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Clustered arrays of nucleic acid molecules may be produced using techniques generally known in the art. By way of example, WO 98/44151 and WO 00/18957 both describe methods of nucleic acid amplification which allow amplification products to be immobilised on a solid support in order to form arrays comprised of clusters or "colonies" of immobilised nucleic acid molecules. A further method for the preparation of clustered arrays on a solid-support bound hydrogel is described in further detail below and in the accompanying examples. It will be appreciated that the arrays of the invention, in all aspects, may be arrays of clusters of molecules. Such arrays are a preferred embodiment of all aspects of the invention.

The support for the molecular array (hydrogel-based or otherwise) which may be modified according to the invention is not limited to a particular matrix or substrate. Supports which may be of use in the practise of this invention are as described above.

Some supports to which biomolecules, such as polynucleotides, are attached are silica-based supports themselves. In certain embodiments of the invention these may be covalently modified in some way so as to allow covalent attachment of either polynucleotides, or to immobilise a chemically reactive group hydrogel or a partially formed hydrogel (e.g. a prepolymer). The surface-activating agent is typically an organosilicon (organosilane) compound such as those listed above.

Arrays in which polynucleotides have been directly attached to silica-based supports are those for example disclosed in WO 97/04131, wherein hairpin polynucleotides are immobilised on a glass support by reaction between a pendant epoxide group on the glass with an internal amino group held within the loop.

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Zhao *et al.* (Nucleic Acids Research, 2001, **29**(4), 955-959) disclose the formation of a hairpin polynucleotide which contains multiple phosphorothioate moieties in the loop. The moieties are used to anchor, in more than one position, the hairpin DNA to glass slides pre-activated with bromoacetamidopropylsilane.

The work of Zhao developed upon earlier work of Pirrung *et al.* (*Langmuir*, 2000, **16**, 2185-2191) in which the authors report that 5'-thiophosphate-terminating oligonucleotides could be attached to glass, pre-activated with mono- and dialkoxylated silanes and bromoacetamide.

In addition, we disclose in our copending International patent application number PCT/GB2004/004707 arrays of hairpin polynucleotides attached to a solid support, e.g. for use in the preparation of SMAs, by reaction of a sulfur-based nucleophile with the solid support. The sulfur-based nucleophile may be directly attached to the hairpin although it is preferably indirectly attached through a linker. Attachment is by way of an internal nucleotide within the hairpin, that is to say that the sulfur-based nucleophile is not connected directly or through a linker to a nucleotide at either terminus of the hairpin.

Still further example of arrays are those in which biomolecules, preferably polynucleotides, are attached to hydrogels supported upon silica-based or other solid supports. Silica-based supports are typically used to support hydrogels and hydrogel arrays as described in WO00/31148, WO01/01143, WO02/12566, WO03/014392, US Patent No. 6,465,178 and WO00/53812.

The solid supports for such hydrogels are preferably silica-based since the silca-based support need not be covalently modified by preactivation with a silylating agent as described above in order to immobilise the hydrogel thereto. Clearly, however, such hydrogels may still be attached to silica-based supports which have been surface-

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activated, e.g. with an organosilane molecule as described above.

A further type of molecular array which may be treated according to this invention are PEM-supported molecular arrays of the type described by Braslavsky et al. (infra), and Kartlov et al. (infra).

There are thus three main types of molecular array which may be treated according to this invention:

(1) arrays directly supported onto silica-based supports;

(2) hydrogel-based molecular arrays; and

(3) PEM-supported molecular arrays.

Of these the hydrogel-based molecular arrays are most preferred, and in particular hydrogel-based arrays according to the fourth aspect of the invention primarily because of the simplicity with which these may be constructed. Whilst, such hydrogels are advantageous on account of the passivity of the surface, we have found that the surface treatment of this invention leads to still greater passivity, and thus utility in sequencing reactions and the like, of the resultant molecular arrays.

According to this invention, the surface of an existing array of biomolecules, preferably of polynucleotides, is modified by treatment with a mixture comprising polyelectrolyte a mixture comprising neutral polymers, or a mixture comprising both polyelectrolytes and neutral polymers.

Polyelectrolytes are large, generally polymeric, molecules containing a plurality of ionisable groups. Examples include polyallylamine (PAL), commercially available as polyallylamine hydrochloride (PAL.HCl), polyacrylic acid (PAA), poly(styrene sulfonate) (PSS) and polyethyleneimine (PEI). The degree to which they are ionised is dependent upon the pH of the medium in which they are present.

In the context of DNA sequencing, we have found that a combination of more than one polyelectrolyte is particularly advantageous when modifying molecular arrays. As an example, we have found that the sequential application of
5   PAL.HCl followed by PAA to be particularly preferred.

The conditions under which the arrays may be treated include exposing them to solutions, or suspensions of polyelectrolyte or neutral polymer. Preferably these solutions or suspensions are aqueous. Particularly
10   preferably the pH of the solution or suspension is higher than 6 and less than 8.5, more preferably from 6.5 to 8, still more preferably from 6.5 to 7.5, more preferably approximately neutral, or about pH 7.

As an alternative to polyelectrolytes, neutral
15   polymers, e.g. polyethylene glycols, such as those commercially available, e.g. PEG 8000 available from Sigma, may be used.

Of course both polyelectrolyte and neutral polymers may be used.
20   It will be appreciated that the treatment of molecular arrays according to this invention shall generally, particularly in the case of planar arrays, serve to deposit layers of polyelectrolyte and/or polymers.

25   When polynucleotides are arrayed in molecular arrays according to the various aspects of this invention, including the solid-supported hydrogel-based molecular arrays of the invention, these are preferably hairpin polynucleotides comprising a polynucleotide duplex which may
30   be used to retain a primer and a target polynucleotide in spatial relationship. Preferably the target polynucleotide is present at the 5' end and the primer is present at the 3' end although hairpin polynucleotides where the primer is present at the 5' end and the target polynucleotide is
35   present at the 3' end are also embraced by this invention.

As used herein, the term "interrogate" can refer to any interaction of a molecule on the array with any other chemical or molecule and may also refer to any analysis of a detectable signal from a molecule on the array or any other
5   molecule which is bound thereto or associated therewith. In one embodiment "interrogation" encompasses a target polynucleotide on the array functioning as a template upon which DNA polymerase acts. In other words, "interrogating" can encompass contacting the target polynucleotides with
10   another molecule, e.g., a polymerase, a nucleoside triphosphate, a complementary nucleic acid sequence, wherein the physical interaction provides information regarding a characteristic of the arrayed target polynucleotide. The contacting can involve covalent or non-covalent interactions
15   with the other molecule. As used herein, "information regarding a characteristic" means information about the identity or sequence of one or more nucleotides in the target polynucleotide, the length of the polynucleotide, the base composition of the polynucleotide, the T_m of the
20   polynucleotide, the presence of a specific binding site for a polypeptide, a complementary nucleic acid or other molecule, the presence of an adduct or modified nucleotide, or the three-dimensional structure of the polynucleotide.

The spatial relationship between primer and target
25   polynucleotide present in hairpin polynucleotides permits improved sequence analysis procedures to be conducted. Maintenance of the spatial relationship is made possible not only by the hydrogen bonds formed on hybridisation, but also by the tethering of a known primer to the target
30   polynucleotide. The fixing of the primer, as part of the hairpin structure, to the hydrogel support, ensures that the primer is able to perform its priming function during a polymerase-based sequencing procedure, and is not removed during any washing step in the procedure.

There are many different ways of forming a hairpin
structure so as to incorporate the target polynucleotide. A
preferred method is to form a first molecule (which may
contain a non-backbone sulfur-based nucleophile attached
5   through a linker) capable of forming a hairpin structure,
and ligate the target polynucleotide to this. It is
possible to ligate any desired target polynucleotide to the
hairpin construct before or after arraying the hairpins on
the solid support. Alternatively, a first polynucleotide
10   may be ligated before arraying and a second ligated after
arraying. It is, of course, also possible to introduce a
nucleophile (preferably a sulfur-based nucleophile) after
such a ligation.

        Where a target polynucleotide is a double-stranded DNA,
15   this may be attached to the stem of the hairpin by ligating
one strand to the hairpin polynucleotide and removing the
other strand after the ligation.

        The target polynucleotide may be genomic DNA purified
using conventional methods. The genomic DNA may be PCR-
20   amplified or used directly to generate fragments of DNA
using either restriction endonucleases, other suitable
enzymes, a mechanical form of fragmentation or a non-
enzymatic chemical fragmentation method. In the case of
fragments generated by restriction endonucleases, hairpin
25   structures bearing a complementary restriction site at the
end of the first hairpin may be used, and selective ligation
of one strand of the DNA sample fragments may be achieved by
one of two methods.

        Method 1 uses a hairpin containing a phosphorylated 5'
30   end. Using this method, it may be necessary to first de-
phosphorylate the restriction-cleaved genomic or other DNA
fragments prior to ligation such that only one sample strand
is covalently ligated to the hairpin.

        Method 2: in the design of the hairpin, a single (or
35   more) base gap can be incorporated at the 3' end (the

receded strand) such that upon ligation of the DNA fragments only one strand is covalently joined to the hairpin. The base gap can be formed by hybridising a further separate polynucleotide to the 5'-end of the first hairpin structure.

5   On ligation, the DNA fragment has one strand joined to the 5'-end of the first hairpin, and the other strand joined to the 3'-end of the further polynucleotide. The further polynucleotide (and the other stand of the fragment) may then be removed by disrupting hybridisation.

10   -   In either case, the net result should be covalent ligation of only one strand of a DNA fragment of genomic or other DNA to the hairpin. Such ligation reactions may be carried out in solution at optimised concentrations based on conventional ligation chemistry, for example, carried out by

15   DNA ligases or non-enzymatic chemical ligation. Should the fragmented DNA be generated by random shearing of genomic DNA or polymerase, then the ends can be filled in with Klenow fragment to generate blunt-ended fragments which may be blunt-end-ligated onto blunt-ended hairpins.

20   Alternatively, the blunt-ended DNA fragments may be ligated to polynucleotide adapters which are designed to allow compatible ligation with the sticky-end hairpins, in the manner described hereinbefore.

25   Polynucleotides, particularly hairpin polynucleotides, may be bound directly to the "C" groups of hydrogels, if present, by, for example, immobilising them through a covalent bond between each polynucleotide (by way of a nucleophile, preferably sulfur-based nucleophile) and the

30   "C" group. In doing so it is thus possible to generate arrays, e.g. microarrays or SMAs, preferably SMAs, of the hairpin polynucleotides.

          The precise density of the arrays is not critical. For single molecule resolution, in fact, the higher the density

35   of hairpin polynucleotide molecules arrayed the better since

more information may be obtained from any one experiment.
For example, there may be at least 10^3 molecules/cm^2,
preferably at least 10^5 molecules/cm^2 and most preferably
10^6-10^9 molecules/cm^2. Particularly preferably, the density
5   of sample molecules is at least 10^7/cm^2, typically it is
approximately 10^8-10^9 /cm^2.

        Such "high density" arrays are in contrast to those
arrays such as those so described in the prior art which are
not necessarily as high or, e.g. in the many molecule arrays
10   of Fodor et al (supra), which comprise clusters of
polynucleotides comprising a plurality of tightly packed
polynucleotides that are resolvable at the level of the
cluster, not at the level of the polynucleotides, are too
high to allow single molecule resolution. By arraying the
15   polynucleotides at a density that they can be considered to
be single molecules, i.e. each can be individually resolved,
a SMA is created.

        The terms "individually resolved" and "individual
resolution" are used herein to specify that, when
20   visualised, it is possible to distinguish one molecule on
the array from its neighbouring molecules. Separation
between individual molecules on the array will be
determined, in part, by the particular technique used to
resolve the individual molecules. It will usually be the
25   target polynucleotide portion that is individually resolved,
as it is this component which is intended to be
interrogated, e.g. by the incorporation of detectable bases.

        Covalent bonding, where present, between the "C" groups
in the hydrogel and polynucleotides (e.g. hairpin
30   polynucleotides or oligonucleotide primers used for
formation of clustered arrays by solid-phase amplification),
may be effected by any convenient means.

        Preferably, where the "C" group is a haloacetamido
group, polynucleotides bearing sulfur-containing
35   nucleophilic groups are used. Examples of appropriate

sulfur nucleophile-containing polynucleotides are disclosed in *Zhao et al* (*Nucleic Acids Research*, 2001, **29**(4), 955-959) and *Pirrung et al* (*Langmuir*, 2000, **16**, 2185-2191).

However, the preferred class of sulfur-based

5   nucleophiles pendant from the polynucleotides for reaction with the reactive groups on the hydrogels are not particularly restricted. The sulfur-based nucleophile may thus be a simple thiol (~SH wherein ~ denotes the bond or linker connecting the thiol to the remainder of the

10 polynucleotide). Further examples of sulfur-based nucleophiles include a moiety of the formula (III):

$$\sim X - \overset{\overset{\displaystyle Y}{\|}}{\underset{\underset{\displaystyle Z}{|}}{P}} - S \qquad \text{(III)}$$
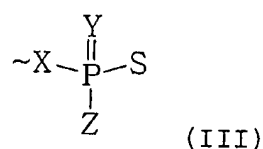
15 (wherein ~ denotes the bond or linker connecting the sulfur-based nucleophile to the remainder of the polynucleotide; X represents an oxygen atom, a sulfur atom or a group NR, in which R is hydrogen or an optionally substituted C_{1-10} alkyl; Y represents an oxygen or a sulfur atom; and Z represents an

20 oxygen atom, a sulfur atom or an optionally substituted C_{1-10} alkyl group).

Preferred moieties of formula (III) are those in which X is oxygen or sulfur, preferably oxygen. Where X is a group NR, R is preferably hydrogen. Y is preferably oxygen.

25 Z is preferably an oxygen or sulfur atom or a methyl group, particularly preferably an oxygen atom.

The preferred sulfur-based nucleophile is thiophosphate although other sulfur-based nucleophiles described are also of utility, for example thiophosphoramidates.

30   Particularly preferably, the sulfur-containing nucleophiles described herein are connected to the polynucleotide through a linker group. The linker may be a

carbon-containing chain such as those of formula $(CH_2)_n$
wherein "n" is from 1 to about 1500, for example less than
about 1000, preferably less than 100, e.g. from 2-50,
particularly 5-25. However, a variety of other linkers may
be employed with the only restriction placed on their
structures being that the linkers are stable under
conditions under which the polynucleotides are intended to
be used subsequently, e.g. conditions used in DNA
sequencing.

Linkers which do not consist of only carbon atoms may
also be used. Such linkers include polyethylene glycol
(PEG) having a general formula of $(CH_2-CH_2-O)_m$, wherein m is
from about 1 to 600, preferably less than about 500.

Linkers formed primarily from chains of carbon atoms
and from PEG may be modified so as to contain functional
groups which interrupt the chains. Examples of such groups
include ketones, esters, amines, amides, ethers, thioethers,
sulfoxides, sulfones. Separately or in combination with the
presence of such functional groups may be employed alkene,
alkyne, aromatic or heteroaromatic moieties, or cyclic
aliphatic moieties (e.g. cyclohexyl). Cyclohexyl or phenyl
rings may, for example, be connected to a PEG or $(CH_2)_n$
chain through their 1- and 4-positions.

Examples of appropriately modified linkers are those of
formula $(CH_2)_n$ (wherein n is as defined above) and in which
one or more CH_2 units are replaced with functional groups).
Thus, one or more CH_2 units may be exchanged for an oxygen
to form an ether, or for a SO_2 to form a sulfone etc. One
or more CH_2 units may be exchanged for an amide moiety or
alkene or alkyne unit. In such linkers one or more
functional groups may be present; these functional groups
may or may not be the same as each other.

Linkers of particular interest contain the
propargylamino unit attached to the base (e.g. uracil) in a

modified nucleotide. Such nucleotides contain the following
unit:
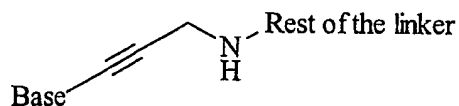
5   The amino group may be connected to the remainder of
the linker by formation of an amide bond.

        Modified nucleotides are commercially available, e.g.
from the DNA synthesis company Oswel (now Eurogentec Group).
Such nucleotides include 3'OH capped nucleotides which may
10   be abasic where a capped linker is attached at the 1'carbon
atom or contain a base to which a capped linker is attached.
Two such modified nucleotides are Oswel products OSW428 and
OSW421:

OSW428

OSW421
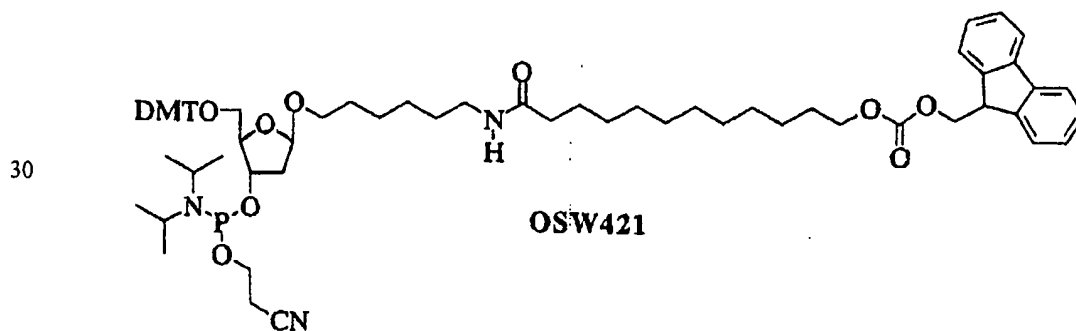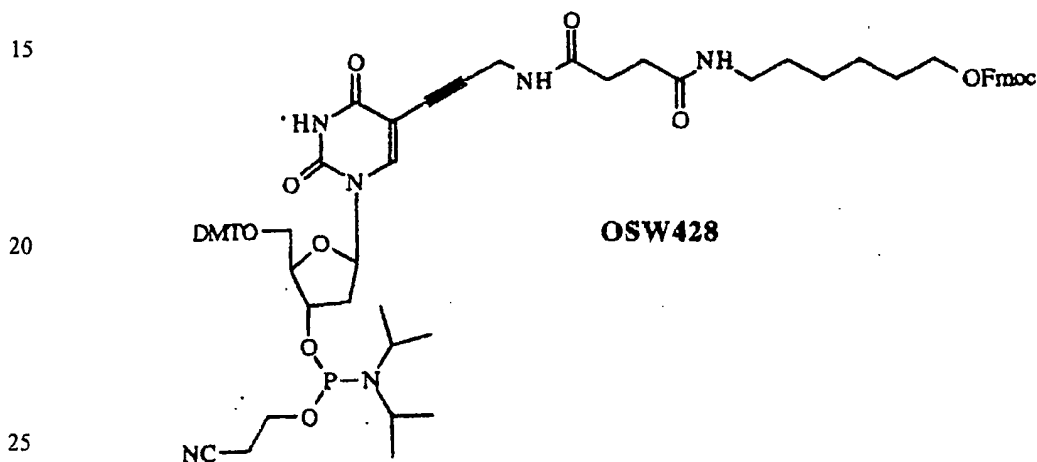
Those skilled in the art will be aware of methods of
deprotecting the fluorenylmethoxycarbonyl (Fmoc) group which
caps the linker in the nucleotides shown above and for
5   effecting terminal modification, e.g. thiophosphorylation,
of the linker.

As an alternative to the linkers described above, which
are primarily based on linear chains of saturated carbon
atoms, optionally interrupted with unsaturated carbon atoms
10 or heteroatoms, other linkers may be envisaged which are
based on nucleic acids or monosaccharide units (e.g.
dextrose). It is also within the scope of this invention to
utilise peptides as linkers.

15 Longer linker moieties (e.g. those containing a chain
or more than 100 atoms, particularly those in excess of 500
or even 1000 atoms) serve to position the polynucleotide
further away from the solid support. This places the
polynucleotide (e.g. DNA) in a environment having a greater
20 resemblance to free solution. This can be beneficial, for
example, in enzyme-mediated reactions effected on the
polynucleotide. This is because such reactions suffer less
from the steric hindrance which manifests itself where the
polynucleotide is directly attached to the support or is
25 indirectly attached through a very short linker (such as a
linker comprising a chain or only several, e.g. about 1 to 3
carbon atoms).

Where the molecules of interest immobilised on the
array are polynucleotides the "linker" may comprise one or
30 more nucleotides which form part of the polynucleotide but
which do not participate in any reaction carried out on or
with the polynucleotide (e.g. a hybridisation or
amplification reaction). Such nucleotides may be referred
to herein as "spacer" polynucleotides. Typically from 1 to
35 20, more preferably from 1 to 15 or from 1 to 10, and more

particularly 2, 3, 4, 5, 6, 7, 8, 9 or 10 spacer nucleotides
may be included. Most preferably the polynucleotide will
include 10 spacer nucleotides. It is preferred to use polyT
spacers, although other nucleotides and combinations thereof

5   can be used. In the most preferred embodiment the
polynucleotide will include 10T spacer nucleotides.

Spacer nucleotides are typically included at the 5'
ends of polynucleotides which are attached to a suitable
support, for example a solid-supported hydrogel, via a

10   linkage with the 5' end of the polynucleotide. Attachment
can be achieved through a sulphur-containing nucleophile,
such as phosphorothioate, present at the 5' end of the
polynucleotide. In the case of arrays based on solid-
supported hydrogels, this nucleophile is bound to a "C"

15   group present in the hydrogel. The one or more spacer
nucleotides function to space the portions of the
polynucleotide that will be "interrogated" and/or subject to
further manipulations, such as hybridisation to a second
polynucleotide, away from the site of attachment to the

20   solid support. The present inventors have observed that
inclusion of spacer nucleotides at the 5' end can markedly
improve the performance of hybridisation of complementary
polynucleotides to target regions of the immobilised
polynucleotides downstream (3') of the spacer nucleotides.

25   Hybridisation yield is observed to increase sharply with
polyT spacers of from 2 to 10T nucleotides. When spacer
length is increased from 10T up to 20T hybridisation yield
begins to decrease. In the most preferred embodiment the
polynucleotide will include 10T spacer nucleotides and a 5'

30   phosphorothioate group.

Whilst the use of sulfur nucleophile-containing
polynucleotides (particularly thiophosphate-containing)and
haloacetamide C groups are preferred to effect attachment of
polynucleotides in all aspects of the invention, the skilled

35   person will be able to envisage many other combinations of

functionality which will facilitate immobilisation of
polynucleotides to the hydrogels described herein. For
example, the C group may comprise an activated ester and the
polynucleotide may bear an amino group or an oxygen-based
5   nucleophile. Other combinations will be evident to those
skilled in the art.

In a particular embodiment the invention provides a
method of forming a clustered array on a solid-supported
hydrogel by means of a nucleic acid amplification reaction.
10   · Therefore, in a further aspect the invention provides a
method of preparing a solid supported hydrogel-based
molecular array which is a clustered array of molecules of
interest, the method comprising:

15   (i) reacting polynucleotide molecules with reactive sites
present in a solid-supported hydrogel, wherein said
polynucleotide molecules are first and second
oligonucleotide primers capable of hybridising to a template
to be amplified;

20

(ii) contacting the first oligonucleotide primers attached
to the solid-supported hydrogel in step (i) with one or more
templates to be amplified under conditions which permit
hybridisation of the templates to the oligonucleotide
25   primers, each template comprising at the 3' end a sequence
capable of hybridising to the first oligonucleotide primer
and at the 5' end a sequence the complement of which is
capable of hybridising to a second oligonucleotide primer;
and

30

(iii) performing one or more nucleic acid amplification
reactions using the first and second oligonucleotide primers
and the template(s), thereby generating a clustered array of
molecules of interest.

35

In this method a solid-supported hydrogel is provided,
preferably prepared using the method according to the first
aspect of the invention. Oligonucleotide primers are then
linked or "grafted" to the hydrogel via reaction with
5   reactive sites present on the hydrogel. This step can be
carried out as described above and all preferred features
described apply *mutatis mutandis* to this aspect of the
invention. The oligonucleotide primers are preferably
attached to the hydrogel via covalent linkage their 5' ends
10 leaving the 3' end of the molecule free to participate in
hybridisation to a template polynucleotide and subsequent
primer extension by addition of further nucleotides to the
free 3' end of the primer. Attachment could also be
effected via an internal nucleotide in the primer, provided
15 that this does not prevent the primer from hybridising to a
template and subsequent primer extension. The most
preferred means of attachment is via 5' phosphorothioate to
a hydrogel comprised of polymerised acrylamide and BRAPA.
     The precise sequence of the primer oligonucleotides
20 will be dependent on the nature of the template it is
intended to amplify. The first and second primers may be of
different or identical sequence. The primers can include
natural and non-natural bases or any combination thereof,
and may also include non-natural backbone linkages such as
25 phosphorothioate. The primer may advantageously' include
spacer nucleotides, as described above, in order to optimise
the efficiency of subsequent hybridisation to the template
polynucleotide. The primer may contain from 1 to 20, more
preferably from 1 to 15 or from 1 to 10, and more
30 particularly 2, 3, 4, 5, 6, 7, 8, 9 or 10 spacer
nucleotides. Most preferably the primer will include 10
spacer nucleotides. It is preferred to use polyT spacers,
although other nucleotides and combinations thereof can be
used. In the most preferred embodiment the primer will
35 include 10T spacer nucleotides.

The primer oligonucleotides are grafted onto the
surface of the solid-supported hydrogel, effectively forming
a surface that is ready to be used for nucleic acid
amplification. This approach contrasts with prior art
5 methods for amplification on solid supports, such as that
described in WO 98/44151 (schematically illustrated in
Figure 5), wherein a mixture of primers and templates are
grafted to the solid surface simultaneously in a single
grafting step. In this approach a specific grafting mixture
10 of primers and templates has to be used for each specific
template to be amplified. In addition, grafting of long
template nucleic acid fragments (>300bp) is technically
difficult. The inventors' approach avoids this problem by
removing the need to graft primers and templates
15 simultaneously. In the method of the invention primers are
grafted in the absence of template to form a surface that is
ready for hybridisation to the template and subsequent
amplification.

Following attachment of the primers the solid support
20 is contacted with the template to be amplified under
conditions which permit hybridisation between the template
and the bound primers. The template is generally added in
free solution and suitable hybridisation conditions will be
apparent to the skilled reader. Typically hybridisation
25 conditions are 5xSSC at 40°C, following an initial
denaturation step.

The template polynucleotide (or a denatured single
strand thereof if referring to a template duplex) will
include at the 3' end a sequence which permits hybridisation
30 to a first oligonucleotide primer and at the 5' end of the
same strand a sequence, the complement of which permits
hybridisation to a second oligonucleotide primer (i.e. the
sequence of the second primer may be substantially identical
to the sequence at the 5' end of the template). The
35 remainder of the template can be any polynucleotide molecule

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that it is desired to amplify to form a clustered array.
Templates may be, for example, fragments of genomic DNA or
cDNA that it is desired to sequence. The sequences
permitting hybridisation to primers will typically be around
5   20-25 nucleotides in length. The term "hybrisidation"
encompasses sequence-specific binding between primer and
template. Binding of the primer to its cognate sequence in
the template can occur under typical conditions used for
primer-template annealing in standard PCR.

10   · Following hybridisation of the templates to primers
bound to the solid support a nucleic acid amplification
reaction can be carried out using the bound primers and the
hybridised template. The first step of the amplification
reaction will be a primer extension step, in which
15   nucleotides are added to the free 3' ends of the bound
primers in order to synthesise complementary strands
corresponding to the full length of the template
(illustrated schematically in Figure 6). Subsequent
denaturation results in a full-length complementary template
20   strand covalently bound to the solid support. This
complementary strand will thus include at its 3' end a
sequence which is capable of binding to the second
oligonucleotide primer. Further rounds of amplification
(analogous to a standard PCR reaction) lead to the formation
25   of clusters or colonies of template molecules bound to the
solid support.

     DNA amplification on solid supports is a procedure well
documented in the literature. A wide range of support types
(e.g. microarrays (Huber M. et al. (2001) Anal. Biochem.
30   299(1), 24-30; Rovera G. (2001) US patent 6221635 B1
20010424), glass beads (Adessi C. et al. (2000) Nucl. Acids
Res. 28(20), e87; Andreadis J. D. et al. (2000) Nucl. Acids
Res. 28(2), e5), agarose (Stamm S. et al. (1991) Nucl.
Acids Res. 19(6), 1350) or polyacrylamide (Shapero M. H. et
35   al. (2001) Genome Res. 11,1926-1934; Mitra, R. D. et al.

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(1999) Nucl. Acids Res. 27(24), e34)) and attachment
chemistries (e.g. 5'-thiol oligo on aminosilane slides via
heterofunctional crosslinker (Adessi C. et al. (2000) Nucl.
Acids Res. 28(20), e87; Andreadis J. D. et al. (2000) Nucl.

5   Acids Res. 28(2), e5), EDC chemistry on NucleoLink™ surface
(Sjoroos M. et al. (2001) Clin. Chem. 47(3), 498-504) or
amino silane (Adessi C. et al. (2000) Nucl. Acids Res.
28(20), e87), radical polymerization (Shapero M. H. et al.
(2001) Genome Res. 11,1926-1934; Mitra, R. D.et al. (1999)

10 Nucl. Acids Res. 27(24), e34)) have been described. PCR on
polyacrylamide coated glass slides (Shapero et al., ibid) or
beads (Mitra et al., ibid) has also been reported. In both
cases, at least one of the primers contains a 5'-acrylamide
modification so that the primer is covalently attached to

15 the solid support through copolymerization. The method of
Mitra et al. consists in premixing of all reagents necessary
to perform the PCR, the primers and a very low concentration
of the template, then polymerizing a thin polyacrylamide
film onto glass slides. In the method of Shapero et al., the

20 primers are both copolymerized while coating the beads and
the template is introduced by hybridization later on.

       One of the major drawbacks of both methods is the lack
of versatility of the surface because of the introduction of
the primers with or without the template during preparation

25 of the surface. Moreover, the primers or the template can
be potentially damaged by the free radicals generated during
the polyacrylamide polymerization.

       WO 98/44151 describes the use of a hybridisation
technique of a template to a primer followed by a chain

30 extension and then replication by PCR to generate colonies
or clusters of immobilised nucleic acids on a solid support.
The cluster technology as described in WO 98/44151 involves
a grafting step that consists of immobilizing simultaneously
both primers and templates on a carboxylated surface using

standard coupling (EDC) chemistry. This attachment is
covalent and has to be done using a specific reaction
mixture for every template used. The strategy used is
illustrated in Figure 5.

5   The inventors' approach involves an initial step of
preparing the surface of the solid support, followed by
covalent attachment (grafting) of primers to generate a
surface ready for use in PCR. The PCR template may then be
hybridised to attached primers immediately prior to the PCR
10   reaction. The PCR reaction thus begins with an initial
primer extension step rather than template denaturation.
This approach is illustrated in Figure 6.

Uses of arrays prepared according to the invention

15   Once formed, arrays prepared according to the invention
may be used in essentially any method of analysis which
requires interrogation of molecules of interest on the array
or of molecules bound to molecules of interest on the array.
In this context, molecules "bound" to molecules of interest
20   on the array includes complementary polynucleotide strands
hybridised to polynucleotides bound on the array. By way of
example, the arrays may be used to determine the properties
or identities of cognate molecules. Typically, interactions
between biological or chemical molecules with molecules of
25   interest bound on the arrays are carried out in solution.
In preferred embodiments the arrays may be used in
procedures to determine the sequence of polynucleotides on
the array, and also in the identification and/or scoring of
single nucleotide polymorphisms, gene expression analysis,
30   etc.
       In particular, the arrays may be used in assays which
rely on the detection of fluorescent labels to obtain
information on the arrayed molecules, typically arrayed
polynucleotides. The arrays are particularly suitable for

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use in multi-step assays. The arrays may be used in
conventional techniques for obtaining genetic sequence
information. Many of these techniques rely on the stepwise
identification of suitably labelled nucleotides, referred to
5   in US Patent No. 5,654,413 as "single base" sequencing
methods or "sequencing-by-synthesis".

In an embodiment of the invention, the sequence of a
target polynucleotide is determined in a similar manner to
that described in US Patent No. 5,654,413, by detecting the
10   incorporation of one or more nucleotides into a nascent
strand complementary to the target polynucleotide to be
sequenced through the detection of fluorescent label(s)
attached to the incorporated nucleotide(s). Sequencing of
the target polynucleotide is primed with a suitable primer
15   (or prepared as a hairpin construct which will contain the
primer as part of the hairpin), and the nascent chain is
extended in a stepwise manner by addition of nucleotides to
the 3' end of the primer in a polymerase-catalysed reaction.

In preferred embodiments each of the different
20   nucleotides (A, T, G and C) is labelled with a unique
fluorophore which acts as a blocking group at the 3'
position to prevent uncontrolled polymerisation. The
polymerase enzyme incorporates a nucleotide into the nascent
chain complementary to the target polynucleotide, and the
25   blocking group prevents further incorporation of
nucleotides. The array surface is then cleared of
unincorporated nucleotides and each incorporated nucleotide
is "read" optically by suitable means, such as a charge-
coupled device using laser excitation and filters. The 3'-
30   blocking group is then removed (deprotected), to expose the
nascent chain for further nucleotide incorporation.

Similarly, US Patent No. 5,302,509 discloses a method
to sequence polynucleotides immobilised on a solid support.
The method relies on the incorporation of fluorescently-
35   labelled, 3'-blocked nucleotides A, G, C and T into a

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growing strand complementary to the immobilised polynucleotide, in the presence of DNA polymerase. The polymerase incorporates a base complementary to the target polynucleotide, but is prevented from further addition by

5   the 3'-blocking group. The label of the incorporated base can then be determined and the blocking group removed by chemical cleavage to allow further polymerisation to occur.

In the case of single molecule arrays, because the array consists of distinct optically resolvable

10   polynucleotides, each target polynucleotide will generate a series of distinct signals as the fluorescent events are detected. The sequence of the target polynucleotide is inferred from the order of addition of nucleotides in the complementary strand following conventional rules of base-

15   pairing.

The term "individually resolved by optical microscopy" is used herein to indicate that, when visualised, it is possible to distinguish at least one polynucleotide on the array from its neighbouring polynucleotides using optical

20   microscopy methods available in the art. Visualisation may be effected by the use of reporter labels, e.g., fluorophores, the signal of which is individually resolved.

Other suitable sequencing procedures will be apparent to the skilled person. In particular, the sequencing method

25   may rely on the degradation of the arrayed polynucleotides, the degradation products being characterised to determine the sequence.

An example of a suitable degradation technique is disclosed in WO95/20053, whereby bases on a polynucleotide

30   are removed sequentially, a predetermined number at a time, through the use of labelled adaptors specific for the bases, and a defined exonuclease cleavage.

A consequence of sequencing using non-destructive methods is that it is possible to form a spatially

35   addressable array for further characterisation studies, and

therefore non-destructive sequencing may be preferred. In
this context, the term "spatially addressable" is used
herein to describe how different molecules may be identified
on the basis of their position on an array.

5   In the case that the target polynucleotide fragments
are generated via restriction digest of genomic DNA, the
recognition sequence of the restriction or other nuclease
enzyme will provide 4, 6, 8 bases or more of known sequence
(dependent on the enzyme). Further sequencing of between 10
10 and 20 bases on the array should provide sufficient overall
sequence information to place that stretch of DNA into
unique context with a total human genome sequence, thus
enabling the sequence information to be used for genotyping
and more specifically single nucleotide polymorphism (SNP)
15 scoring.

        The sequencing method that is used to characterise the
bound target may be any known in the art that measures the
sequential incorporation of bases onto an extending strand.
A suitable technique is disclosed in US Patent No. 5,302,509
20 requiring the monitoring of sequential incorporation of
fluorescently-labelled bases onto a complement using the
polymerase reaction. Alternatives will be apparent to the
skilled person. Suitable reagents, including fluorescently-
labelled nucleotides will be apparent to the skilled person.

25   Thus the devices into which the arrays of this
invention may be incorporated include, for example, a
sequencing machine or genetic analysis machine.

        In the case of single molecule arrays the single
polynucleotides immobilised onto the surface of a solid
30 support should be capable of being resolved by optical
means. This means that, within the resolvable area of the
particular imaging device used, there must be one or more
distinct signals, each representing one polynucleotide.
Typically, the polynucleotides of the array are resolved
35 using a single molecule fluorescence microscope equipped

with a sensitive detector, e.g., a charge-coupled device (CCD). Each polynucleotide of the array may be imaged simultaneously or, by scanning the array, a fast sequential analysis can be performed.

The extent of separation between the individual polynucleotides on the array will be determined, in part, by the particular technique used to resolve the individual polynucleotide. Apparatus used to image molecular arrays are known to those skilled in the art. For example, a confocal scanning microscope may be used to scan the surface of the array with a laser to image directly a fluorophore incorporated on the individual polynucleotide by fluorescence. Alternatively, a sensitive 2-D detector, such as a charge-coupled device, can be used to provide a 2-D image representing the individual polynucleotides on the array.

"Resolving" single polynucleotides on the array with a 2-D detector can be done if, at 100 x magnification, adjacent polynucleotides are separated by a distance of approximately at least 250 nm, preferably at lest 300 nm and more preferably at least 350 nm. It will be appreciated that these distances are dependent on magnification, and that other values can be determined accordingly, by one of ordinary skill in the art.

Other techniques such as scanning near-field optical microscopy (SNOM) are available which are capable of greater optical resolution, thereby permitting more dense arrays to be used. For example, using SNOM, adjacent polynucleotides may be separated by a distance of less than 100 nm, e.g., 10 nm. For a description of scanning near-field optical microscopy, see Moyer et al., *Laser Focus World* (1993) 29(10).

An additional technique that may be used is surface-specific total internal reflection fluorescence microscopy (TIRFM); see, for example, Vale et al., *Nature* (1996)

380:451-453). Using this technique, it is possible to achieve wide-field imaging (up to 100 μm x 100 μm) with single molecule sensitivity. This may allow arrays of greater than 10^7 resolvable polynucleotides per cm^2 to be
5   used.

    Additionally, the techniques of scanning tunnelling microscopy (Binnig et al., Helvetica Physica Acta (1982) 55:726-735) and atomic force microscopy (Hansma et al., Ann. Rev. Biophys. Biomol. Struct. (1994) 23:115-139) are
10   suitable for imaging the arrays of the present invention. Other devices which do not rely on microscopy may also be used, provided that they are capable of imaging within discrete areas on a solid support.

    Once sequenced, the spatially addressed arrays may be
15   used in a variety of procedures which require the characterisation of individual molecules from heterogeneous populations.

    Polynucleotides bound on clustered arrays may also be used as templates for "sequencing-by-synthesis" reactions in
20   which one or more nucleotides are successively incorporated into growing strands complementary to the target polynucleotides to be sequenced and the identity of the base(s) added in one or more of the nucleotide incorporation steps is determined. Again sequencing requires a suitable
25   primer complementary to the template which can serve as an initiation point for the addition of further nucleotides in the sequencing reaction. The sequence of the bound polynucleotide is inferred from the identity of the incorporated nucleotides following conventional base-pairing
30   rules. Methods for nucleic acid sequencing on clustered arrays or nucleic acid "colonies" are described, for example, in WO 98/44152, WO 98/44151, WO 00/18957 and WO 03/074734.

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The invention may be understood with reference to the following examples which are to be understood as illustrative, and not limiting, of the present invention.

5 **Preparation 1: Synthesis of *N*-(5-bromoacetamidylpentyl) acrylamide (BRAPA)**

(1)
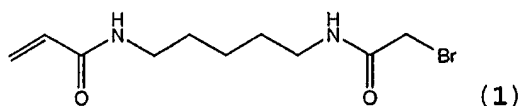
10 *N*-Boc-1,5-diaminopentane toluene sulfonic acid was obtained from Novabiochem. The bromoacetyl chloride and acryloyl chloride were obtained from Fluka. All other reagents were Aldrich products.


15   (2)
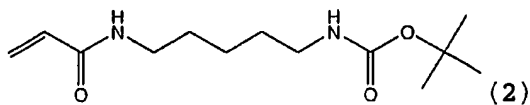
To a stirred suspension of *N*-Boc-1,5-diaminopentane toluene sulfonic acid (5.2 g, 13.88 mmol) and triethylamine (4.83 ml, 2.5 eq) in THF (120 ml) at 0°C was added acryloyl
20 chloride (1.13 ml, 1 eq) through a pressure equalized dropping funnel over a one hour period. The reaction mixture was then stirred at room temperature and the progress of the reaction checked by TLC (petroleum ether : ethyl acetate 1:1). After two hours, the salts formed during the reaction
25 were filtered off and the filtrate evaporated to dryness. The residue was purified by flash chromatography (neat petroleum ether followed by a gradient of ethyl acetate up to 60%) to yield 2.56 g (9.98 mmol, 71 %) of product 2 as a beige solid. ^{1}H NMR (400 MHz, d$_6$-DMSO) : 1.20-1.22 (m, 2H,
30 CH$_2$), 1.29-1.43 (m, 13H, tBu, 2xCH$_2$), 2.86 (q, 2H, J = 6.8 Hz and 12.9 Hz, CH$_2$), 3.07 (q, 2H, J = 6.8 Hz and 12.9 Hz, CH$_2$), 5.53 (dd, 1H, J = 2.3 Hz and 10.1 Hz, CH), 6.05 (dd,

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1H, J = 2.3 Hz and 17.2 Hz, CH), 6.20 (dd, 1H, J = 10.1 Hz

and 17.2 Hz, CH), 6.77 (t, 1H, J = 5.3 Hz, NH), 8.04 (bs,

1H, NH). Mass (electrospray+) calculated for $C_{13}H_{24}N_2O_3$ 256,

found 279 (256+Na^+).

(3)
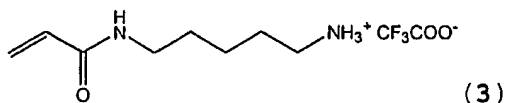
Product **2** (2.56g, 10 mmol) was dissolved in trifluoroacetic

acid:dichloromethane (1:9, 100 ml) and stirred at room

temperature. The progress of the reaction was monitored by

TLC (dichloromethane : methanol 9:1). On completion, the

reaction mixture was evaporated to dryness, the residue co-

evaporated three times with toluene and then purified by

flash chromatography (neat dichloromethane followed by a

gradient of methanol up to 20%). Product **3** was obtained as a

white powder (2.43 g, 9 mmol, 90%). ^{1}H NMR (400 MHz, D_2O):

1.29-1.40 (m, 2H, CH_2), 1.52 (quint., 2H, J = 7.1 Hz, CH_2),

1.61 (quint., 2H, J = 7.7 Hz, CH_2), 2.92 (t, 2H, J = 7.6 Hz,

CH_2), 3.21 (t, 2H, J = 6.8 Hz, CH_2), 5.68 (dd, 1H, J = 1.5

Hz and 10.1 Hz, CH), 6.10 (dd, 1H, J = 1.5 Hz and 17.2 Hz,

CH), 6.20 (dd, 1H, J = 10.1 Hz and 17.2 Hz, CH). Mass

(electrospray+) calculated for $C_8H_{16}N_2O$ 156, found 179

(156+Na^+).

To a suspension of product **3** (6.12 g, 22.64 mmol) and

triethylamine (6.94 ml, 2.2 eq) in THF (120 ml) was added

bromoacetyl chloride (2.07 ml, 1.1eq), through a pressure

equalized dropping funnel, over a one hour period and at

-60°C (cardice and isopropanol bath in a dewar). The

reaction mixture was then stirred at room temperature

overnight and the completion of the reaction was checked by

TLC (dichloromethane : methanol 9:1) the following day. The

salts formed during the reaction were filtered off and the

reaction mixture evaporated to dryness. The residue was
purified by chromatography (neat dichloromethane followed by
a gradient of methanol up to 5%). 3.2 g (11.55 mmol, 51 %)
of the product 1 (BRAPA) were obtained as a white powder. A
further recrystallization performed in petroleum ether:ethyl
acetate gave 3g of the product 1. ^{1}H NMR (400 MHz, d$_6$-DMSO)
: 1.21-1.30 (m, 2H, CH$_2$), 1.34-1.48 (m, 4H, 2xCH$_2$), 3.02-
3.12 (m, 4H, 2xCH$_2$), 3.81 (s, 2H, CH$_2$), 5.56 (d, 1H, J =
9.85 Hz, CH), 6.07 (d, 1H, J = 16.9 Hz, CH), 6.20 (dd, 1H,
J = 10.1 Hz and 16.9 Hz, CH), 8.07 (bs, 1H, NH), 8.27 (bs,
1H, NH). Mass (electrospray+) calculated for C$_{10}$H$_{17}$BrN$_2$O$_2$ 276
or 278, found 279 (278+H$^+$), 299 (276+Na$^+$).

Preparation 2: Preparation of glass support

A: Cleaning of glass slides - The glass slides used for the
preparation of the hydrogel surfaces were cleaned using the
following in-house protocol: the slides were sequentially
incubated in Decon ^{TM}, 1M aqueous sodium hydroxide and
finally 0.1 M hydrochloric acid (aq). After each step, the
slides were sonicated in MilliQ H$_2$O. The cleaned slides were
stored in ethanol.

B: Binder silanization of the glass slides (optional) - The
cleaned glass slides were baked at 120°C for two hours prior
to silanization. After cooling down in a dessicator filled
with argon, the slides were incubated at room temperature in
a 2% v/v solution of either 3-(trimethoxylsilyl)propyl
methacrylate or (3-acryloxypropyl)trimethoxy silane in
toluene HPLC grade. The slides were then rinsed carefully
with toluene and cured for two hours at 120°C. The silanized
slides were stored in a dessicator filled with argon.

Preparation 3: Preparation of the polymerisation mixture

Acrylamide (Purity 99 +%, 0.4 g) was dissolved in MilliQ H_2O
(10 mls) (Solution I). Potassium or ammonium persulfate
(0.25 g) was dissolved in MilliQ H_2O (5 ml) (Solution II).
N-(5- bromoacetamidylpentyl)acrylamide (BRAPA) (33 mg) was
5   dissolved in DMF (330 µl) (Solution III).

Solution I was degassed 10 minutes with argon. Solution III
was added to solution I. After mixing, N, N, N',N'-
tetramethylethylenediamine (TEMED) (23 µl) was added to the
10   mixture. Finally solution II (200 µl) was added. The
polymerisation mixture was rapidly reacted with the
(optionally) silanized slides according to one of the
following protocols. The ratio of BRAPA to acrylamide was 2
mol %.

15

Example 1: Synthesis of polyacrylamide-based surface

**Application of the polyacrylamide hydrogel to the glass
slides**

20
Method I - Two slides (optionally silanised) were assembled
with a silicon gasket in between to form a polymerisation
cell. The slides and the gasket were held together with
binder clips. Polymerisation mixture (800 µl) was injected
25   in each polymerisation cell. The polymerisation proceeded at
room temperature for 1.5 hr. The polymerisation cells were
then disassembled and the slides washed thoroughly under
running MilliQ H_2O. The slides were then dried and stored
under argon.

30
Method II - Slides (optionally silanised) were put into a
clean coplin jar. The polymerisation mixture was poured into
the jar to cover the slides. The polymerisation proceeded at
room temperature for 1.5 hr. The slides were then removed
35   from the coplin jar one by one and rinsed under running
MilliQ H_2O. The slides were then introduced in clean plastic
vials containing MilliQ H_2O and vortexed for 20 seconds. The

slides were rinsed with running MilliQ H_2O, dried and stored under argon.

Example 2: Immobilisation of Polynucleotides on
5   **Polyacrylamide Surfaces**

The following constitutes representative procedures for the immobilisation of 5'-phosphorothioate-modified polynucleotides to the new surface. Oligos with a 3'-
10   fluorescent label are typically used for appraising fundamental surface characteristics.

A: Bulk application (suitable for microarrays)

15   Polynucleotide (1 µM) in the printing buffer (potassium phosphate 100 mM, pH 7) was applied to the surface as 1 µl drops. The slide was then placed in a humid chamber at room temperature for 1 hour. The printed slide was then rinsed with MilliQ H_2O and vortexed in hot washing buffer (Tris HCl
20   10 mM, EDTA 10 mM, pH 8 (at 80-90°C)). The printed slide was finally rinsed with MilliQ H_2O, dried under a flow of argon and stored in the dark before imaging.

To immobilise polynucleotides over a large area, a gasket
25   was placed onto the slide, the polynucleotide solution injected into the chamber formed and a cover slip placed over the gasket. The slide was then incubated for 1 h at room temperature in a humid chamber and processed as described above.
30
B: Single molecule array application

The coated slide was fitted into a custom-made flow cell. Polynucleotide (400 µl, 0.1 to 10 nM) in the printing buffer
35   was injected into the cell. The concentration of polynucleotide is chosen to achieve a precise single molecule density of polynucleotides on the surface. The cell

- 60 -

was incubated in the dark at room temperature for 1 h. The printed surface was then washed by sequentially injecting printing buffer (20 ml), hot washing buffer (20 ml, 80-90°C) and finally MilliQ H_2O (20 ml).

Example 3: Thermal Stability of the Immobilised Polynucleotides

A: Bulk application (suitable for microarrays) - A printed slide was imaged with a fluorescence scanner in the presence of a fluorescence reference control slide containing attached Cy3 dye. The printed slide was then incubated in a jar containing printing buffer at a preset temperature in the dark. The slide was imaged at regular intervals and the fluorescence intensity recorded. The fluorescence intensity of a spot is proportional to the amount of polynucleotide immobilised on that area. A plot of the variation of the fluorescence intensity with time gave a stability profile for attached polynucleotide on the polyacrylamide surface.

B: Single molecule array application - A slide was printed in a flow cell as described above. Printing buffer is injected through the cell at a rate of 1 ml/min, at a preset temperature, and the slide imaged at regular intervals using a custom-made total internal reflection fluorimeter instrument. The stability profile of the single molecule array is obtained by plotting the variation of the number of single molecules counted in a specific area with time.

Example 4: Evaluation of Surface Passivation Towards Nucleotide Sticking at SMA Level

A coated slide was fitted into a custom-made flow cell. The cell was flushed with MilliQ H_2O (10 ml) then phosphate printing buffer (0.1 M, pH 7.0) and then incubated for 30 minutes at room temperature. This procedure constituted a mock DNA couple to the surface. The cell was flushed with

MilliQ H₂O (10 ml), hot TE buffer (10 ml, 10 mM Tris.HCl, 10 mM EDTA, pH 8.0) then MilliQ H₂O (10 ml). The slide was then imaged using a custom-made total internal reflection fluorimeter instrument to give a fluorescent background reading. The cell was then flushed with enzymology buffer (10 ml, 50 mM Tris.HCl, 4mM MgSO₄, 0.2 mM MnCl₂, 0.05% Tween 20, pH 8.0). A solution of fluorescently-labelled nucleotide (400 µl, 0.2 to 2 µM in enzymology buffer) was then injected into the cell and the cell incubated at a preset temperature for 30 minutes. The cell was flushed with wash buffer (10 ml, 50 mM Tris.HCl, 4 mM MgSO₄, 0.05% Tween 20, pH 8.0), high salt buffer (10 ml, 50 mM Tris.HCl, 1M NaCl, 4 mM MgCl₂, 0.05% Tween 20, pH 8.0), TE buffer (10 ml, composition as above) and MilliQ H₂O (10 ml). The slide was then imaged using a custom-made total internal reflection fluorimeter instrument to determine the level of nucleotide sticking to the surface.

Example 5: Evaluation of Enzymology on Surface Immobilised Hairpin DNA

A: Single molecule array application - A coated slide was fitted into a custom-made flow cell. A solution of self-priming DNA hairpin (200 µl, 1 nM, 0.1M KPi, pH 7.0) was then injected into the flow cell and the cell incubated for 1 hr at room temperature. The cell was then flushed with boiling TE buffer (20 ml, 10 mM Tris.HCl, 10 mM EDTA, pH 8.0) and milliQ H₂O (20 ml). The slide was then imaged using a custom-made total internal reflection fluorimeter instrument to give a fluorescent background reading. The flow cell was then flushed with wash buffer (20 ml, 50 mM Tris.HCl, 4 mM MgSO₄, 0.05% Tween 20, pH 8.0) and then enzymology mix (2 x 100 µl, 0.2 µM fluorescently-labelled nucleotide, 5 µg/ml DNA polymerase, 50 mM Tris.HCl, 4 mM MgSO₄, 0.4 mM MnCl₂, 0.05% Tween 20, pH 8.0) was injected

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into the flow cell. The flow cell was incubated at 45°C for
30 minutes. The flow cell was then flushed at a flow rate of
0.5 ml/s with wash buffer (20 ml, 50 mM Tris.HCl, 4 mM
MgSO$_4$, 0.05% Tween 20, pH 8.0), high salt wash buffer (20
5 ml, 50 mM Tris.HCl, 1 M NaCl, 4 mM MgSO$_4$, 0.05% Tween 20, pH
8.0), TE buffer (20 ml, 10 mM Tris.HCl, 10 mM EDTA, pH 8.0)
and milliQ H$_2$O (20 ml). The slide was then imaged using a
custom-made total internal reflection fluorimeter instrument
to determine the level of enzyme incorporation of the
10 fluorescently-labelled nucleotide into the hairpin.

**Example 6: Preparation of a polyelectrolyte-treated fused
silica-supported hydrogel and a demonstration that it is
more passive towards functionalised labelled nucleotides
15 when compared to a non-treated control**

**A: A polyacrylamide-based glass-supported hydrogel is
prepared as described in Example 1.**

20 **B: Pretreatment with polyallylamine hydrochloride followed
by polyacrylic acid**

Treatment of a polyacrylamide hydrogel comprising 1 mol %
BRAPA, as described in Part A, is effected by contacting the
25 hydrogel with a solution of polyallylamine hydrochloride (2
mg/ml) MilliQ H$_2$O at pH 8. Contacting is effected for 30
min at room temperature after which the solution is treated
with polyacrylic acid (2 mg/ml MilliQ H$_2$O at pH 8.2). The
solution is incubated for 30 min at room temperature
30 followed by treatment with MilliQ H$_2$O. The surface treated
with the two layers of polyelectrolyte demonstrates a
reduction in sticking of fluorescently functionalised
nucleotides when compared to a control hydrogel surface not
treated with the polyelectrolytes.

35
**Example 7: Demonstration that of a poly(ethylene glycol)-
treated fused silica-supported hydrogel is more passive**

towards functionalised labelled nucleotides when compared to
a non-treated control

Example 6 was repeated except that instead of treatment with
polyallylamine hydrochloride following by polyacrylic acid,
the hydrogel prepared as in example 1 is treated with
poly(ethylene glycol) 8000 (Sigma). The surface treated
with the poly(ethylene glycol) 8000 demonstrates a reduction
in sticking of fluorescently functionalised nucleotides when
compared to a control hydrogel surface not treated with the
poly(ethylene glycol) 8000.

**Example 8: Demonstration that treatment of a
polyelectrolyte- or poly(ethylene glycol)-treated fused
silica-supported hydrogel-based molecular array is more
passive towards functionalised labelled nucleotides when
compared to a non-treated control**

Examples 6 and 7 are repeated except that instead of
applying the polyelectrolytes or poly(ethylene glycol) to a
fused silica-supported hydrogel as such, instead an array of
polynucleotides is treated and the arrays so modified used
in sequencing reactions with fluorescently labelled
nucleotides. The surfaces treated with either the two
layers of polyelectrolyte, or the poly(ethylene glycol)
8000, demonstrate a reduction in sticking of fluorescently
functionalised nucleotides when compared to a control
hydrogel surfaces not so treated.

Example 9: Preparation of plastics-supported hydrogel

Plastic substrates were obtained from Åmic and were cleaned
with Decon 90 overnight. These were poly(methyl
methacrylate) (Amic PMMA), and cyclic olefin 1060 and 1420
plastics (Amic (COP) 1060 and Amic (COP) 1420). The next
day they were rinsed extensively with MilliQ water and
dried. A 2% w/v acrylamide solution was made up by

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dissolving 1.3 g acrylamide in 65 ml of MilliQ water. This
solution was then purged with argon for 15 minutes to remove
oxygen, which may inhibit the polymerisation reaction.
BRAPA (107 mg) was then dissolved with 1.07 ml of

5   dimethylformamide (DMF) and added to the degassed acrylamide
solution. After mixing, 75 μl of TEMED catalyst was added
to the acrylamide/BRAPA solution. Then polymerisation was
started by adding 0.65 ml of a 0.05 g/ml solution of
potassium persulfate initiator to the acrylamide/BRAPA/TEMED

10 solution. The acrylamide/BRAPA/TEMED/persulfate solution
was quickly mixed and added to a coplin jar containing the
clean dry plastic (and glass) substrates. After 90 minutes
the slides were removed from the polymerising mixture and
rinsed copiously with MilliQ under flow. They were then

15 vortexed 20 seconds in MilliQ and rinsed a second time under
flow before being dried with Argon. Slides treated in this
way are hereinafter referred to as being slides treated with
silane-free acrylamide (SFA) or "support with SFA".

20 **Examples 10: Functionalisation of plastics-supported
    hydrogel**

Following slide preparation as described in Example 9,
CultureWell coverglass gaskets from GraceBio labs were

25 attached to the slides for 1 hour. 1 μM phosphorothioate-
Cy3-DNA (positive) and 1 μM hydroxyl-Cy3-DNA (negative,
control DNA) in 10 μM phosphate buffer pH 7 were then
spotted in wells on the plastic (and glass) slides and
coupling carried out for 1 hour in a humidity chamber at

30 room temperature (20°C). Following coupling each slide was
rinsed copiously with higher ionic strength 0.10 M phosphate
buffer. The gasket was then carefully removed and the slide
then vortexted 20 seconds in 10 mM Tris/10 mM EDTA pH 8
buffer. Finally the slide was rinsed with MilliQ water under

35 flow, then dried with argon.

**Example 11: Dectection of oligonucleotide functionalisation
of plastics-supported hydrogel**

5   Fluorescence scanning was performed on a Typhoon 8600 imager
at 550V, 100 μm resolution in the Cy3 channel (532 laser
excitation).

The images shown in Figure 1 show the attempted coupling of
10 positive (PS, phosphorothioate) and negative (OH, hydroxyl
control) DNA to Åmic PMMA and 1060 and 1420 plastics with
and without SFA. As may be seen by comparison of the 1st
and 2nd columns of images, a fluorescent signal is only
obtained when the plastic has been first coated with SFA.
15 Some non-specific binding of 1 μM hydroxyl DNA is observed
but less than in the case of the glass substrate. This
suggests that substrate effects are still present despite
the presence of SFA coating.

20 The relative levels of positive signal due to
phosphorothioate binding are shown in Figure 2 along with
the measured signal-to-noise values (phosphorothioate
DNA:hydroxyl DNA).

25 The graph shown in Figure 3 shows that the apparent
stability of the specifically adsorbed phosphorothioate DNA
in 50 mM phosphate buffer pH 7 at 65°C is essentially the
same as that for glass. Approximately 40% of the starting
signal is left after 7 days incubation in 50 mM phosphate pH
30 7 at 65°C. This value is slightly lower than expected for
both glass and plastic substrates and is attributed to the
fact that the samples were always scanned dry after briefly
rinsing with MilliQ water. When dry, the fluorescent dye is
greatly affected by environmental conditions, particularly
35 ozone levels.

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Example 12: Further preparation of plastics-supported hydrogels

5   Other plastics (e.g. polystyrene) and plastics of the same
     type but from different suppliers were also tested and
     hybridisation studies attempted as follows:

     Polystyrene from Corning, Zeonex E48R (a cyclic olefin
10   polymer) from Zeon Chemicals Ltd. and poly(methyl
     methacrylate) from the Technical University of Denmark, as
     well as Spectrosil glass substrates, were cleaned as
     described in Example 9. Some clean samples were kept aside
     while others were coated with SFA as described in Example 9.

15
     1 μM phosphorothioate-Cy3-DNA (positive), 1 μM hydroxyl-Cy3-
     DNA (negative, control DNA), 1 μM unlabelled P5 primer with
     a 10T spacer and 1 μM unlabelled P7 primer with a 10T spacer
     were coupled to substrates as prepared in Example 12, and
20   cleaned but not treated with SFA. Scanning was then
     performed as described in Example 12. Following scanning
     hybridisation was carried out using a Texas Red labelled
     complementary target to the P5 primer (P5′) by attaching a
     square silicone gasket to the substrate then placing another
25   clean (uncoated glass) slide on top to form a cell. An
     injection of 0.5 μM complementary target in 5X sodium
     citrate (SSC), 0.1 % Tween 20 buffer pH 7 was then made into
     the space created by the gasket and the cell heated in an
     oven to 95°C for 30 minutes. The oven temperature was then
30   switched to 50°C and the cell allowed to cool for 2 hours.
     Afterwards the cell was removed and allowed to cool at room
     temperature for 10 minutes in the dark. The complementary
     target solution was then removed with a syringe and fresh
     5XSSC, 0.1% Tween 20 buffer at room temperature injected,
35   then removed. This washing procedure was repeated 5 further

times.   The cell was then dismantled and the substate
agitated in a beaker of fresh 5XSSC, 0.1% Tween 20 for 2
minutes.   The substrate was then transferred to a beaker
containing higher stringency (0.1XSSC, 0.1% Tween 20) and
5   agitated for 2 minutes. This was repeated once more then the
substrate dried with argon.   The samples were then scanned
again this time at 700 V in the Rox channel using 633 nm
excitation (100 μm resolution).

10   The images shown in Figure 4 shows below the coupling of the
Cy3-labelled phosphorothioate (GW2) and hydroxyl DNA (GW4)
to glass and plastic substrates with and without SFA
coatings.   Again, the same results were obtained (i.e.
coupling of phosphorothioate DNA only when SFA is present)
15   although the signal intensities and signal-to-noise ratios
vary slightly from before (see graph).

**Example 13: formation of clustered arrays by template
hybridisation and PCR**

20

Overview:

The inventors' approach involves an initial step of
preparing the surface of the solid support, followed by
covalent attachment (grafting) of primers to generate a
25   surface ready for use in PCR.   The PCR template may then be
hybridised to attached primers immediately prior to the PCR
reaction.   The PCR reaction thus begins with an initial
primer extension step rather than template denaturation.
This approach is illustrated in Figure 6.

30

Experimental:

The solid supports used in this experiment were 8-channel
glass chips such as those provided by Micronit (Twente,
Nederland) or IMT (Neuchâtel, Switzerland). However, the
experimental conditions and procedures are readily
5   applicable to other solid supports.

Chips were washed as follows: neat Decon for 30 min, milliQ
H_2O for 30 min, NaOH 1N for 15 min, milliQ H_2O for 30 min,
HCl 0.1N for 15 min, milliQ H_2O for 30 min.

10   Chips were then coated with polyacrylamide hydrogel as
described in Example 1.

5'-phosphorothioate oligonucleotides were grafted onto the
surface of the hydrogel in 10 mM phosphate buffer pH7 for 1h
15   at RT. The following exemplary primers were used :

P7 primer with polyT spacer:

5'phosphorothioate-TTTTTTTTTTCAAGCAGAAGACGGCATACGA -3'

P5 primer with polyT spacer:

20   5'phosphorothioate-TTTTTTTTTTAATGATACGGCGACCACCGA -3'

Primers were used at a concentration of 0.5 μM in the
grafting solution.

25   The template used was a fragment of pBluescript T246
containing sequences complementary to the P7/P5 primers
shown above. The hybridization procedure began with a
heating step in a stringent buffer (95°C for 5 minutes in
TE) to ensure complete denaturation prior to hybridisation
30   of the template. Hybridization was then carried out in 5x

SSC, using template diluted to a final concentration of 5 nM. After the hybridization, the chip was washed for 5 minutes with milliQ water to remove salts.

5   Surface amplification was carried out by thermocycled PCR in an MJ Research thermocycler.

A typical PCR program is as follows:

10

1- 97.5°C for 0:45

2- X°C for 1:30

3- 73°C for 1:30

4- Goto 1 [40] times

15 5- 73°C for 5:00

6- 20°C for 3:00

7- End

Since the first step in the amplification reaction is
20 extension of the primers bound to template in the initial hybridisation step the first denaturation and annealing steps of this program were omitted (i.e. the chip was placed on the heating block only when the PCR mix was pumped through the channels and the temperature was 73°C).

25

The annealing temperature (X°C , step 2) depends on the primer pair that is used. Experiments have determined an optimal annealing temperature of 57°C for P5/P7 primers.

For other primer-pairs the optimum annealing temperature can be determined by experiment. The number of PCR cycles may be varied if required.

5 PCR was carried out in a reaction solution comprising 1x PCR reaction buffer (supplied with the enzyme) 1M betain, 1.3% DMSO, 200 μM dNTPs and 0.025 U/μL Taq polymerase.

In order to visualise colonies the glass chips were stained
10 with SYBR Green-I in TE buffer (1/10 000) and then viewed using an epifluorescence microscope. It was observed that amplified colonies had formed in channels treated as described above, but not in control channels in which the template was added at the primer grafting stage (data not
15 shown).

Example 14: Effect of spacer length on polynucleotide hybridisation

20 **Background:**

DNA microarrays may be used to carry out thousands of heterogeneous (solid-liquid interface) hybridisations simultaneously to determine gene expression patterns or to
25 identify genotype. Hybridisation on these arrays depends on a number of factors including probe density (Peterson, A.W. et al. (2001) Nucl. Acids Res. 29, 5163-5168) while kinetic rates and equilibrium binding constants may differ markedly from the solution phase.

30

Due to steric hindrance and a reduced degree of freedom the
proximity to the support surface is a key criterion
affecting hybridisation yield (Weiler, J et al. (1997) Nucl.
Acids Res. 25, No. 14, 2792-2799). Shchepinov et al.,
determined that 60 atoms was the optimal spacer length for a
hydrophilic PEG-phosphoroamidite synthon spacer (Shchepinov,
M.S. et al. (1997) Nucl. Acids Res. 25, 1155-1161). This
resulted in a 50-fold increase in hybridisation yield.
Above 60 atoms (approx. 10 glycol units) hybridisation yield
decreased until at 30 units the yield of hybridisation was
the same as that with no spacer at all. Moreover, the yield
of hybridisation was affected by the charge density along
the spacer while being independent of charge type.

The hybridisation studies outlined below show that it was
possible to improve hybridisation yield by incorporating a
polyT spacer into the primer. Similar oligoT spacers have
been shown previously to produce a 20-fold enhancement of
hybridization (Guo,Z. et al (1994) Nucl. Acids Res. 22,
5456-5465).

Experimental:

(1)   Hybridization on a microplate-effect of spacer

Modification of glass with silane-free acrylamide (SFA):
A 96 well, glass bottom, polystyrene microplate was placed
into Decon 90 overnight. The next day the microplate was
rinsed extensively with MilliQ water, then dried with Argon.
The glass surface of each well of the microplate was then
coated with silane-free acrylamide (SFA). Briefly,
acrylamide was dissolved in water to give a 2% w/v solution

and argon then bubbled through this solution for 15 minutes.
82.5 mg of BRAPA (the active monomer) was dissolved in 0.825
ml dimethylformamide (DMF). This solution was then added to
50 ml of acrylamide solution to give a 2 mol% BRAPA solution
5   with respect to acrylamide. After mixing 57.5 μl TEMED was
added to the acrylamide/BRAPA solution. A potassium
persulfate solution was then prepared by dissolving 0.1 g in
2 ml MilliQ water. 0.5 ml of the initiator solution was then
added to the degassed acrylamide/BRAPA/TEMED solution and,
10 after mixing, 0.4 ml of the polymerisation mixture was
pipetted into each well of the microplate. Polymerisation
was allowed to proceed for 1.5 hours, then the microplate
was washed on an automated microplate washer with program
AHPEM160. Following washing the plate was dried under argon
15 and stored overnight under vacuum.

Coupling of oligo primers to SFA surface:

The following 4 primers were coupled to the surface at 2.0
μM, 1.0 μM, 0.5 μM and 0.1 μM concentrations from 10 mM
20 phosphate buffer pH 7:

1) P7 primer without polyT spacer:

5'phosphorothioate-CAAGCAGAAGACGGCATACGA -3'

25 2) P7 primer with polyT spacer:

5'phosphorothioate-TTTTTTTTTTCAAGCAGAAGACGGCATACGA -3'

3) P5 primer without polyT spacer:

5'phosphorothioate-AATGATACGGCGACCACCGA -3'

30

4) P5 primer with polyT spacer:

5'phosphorothioate-TTTTTTTTTTAATGATACGGCGACCACCGA -3'

Coupling was carried out using 0.1 ml oligonucleotide
solution for 1 hour in a humid environment at room
temperature. Afterwards the microplate was washed with 0.10
M phosphate buffer pH 7 on an automated microplate washer.

Hybridisation:

Hybridisation to the primers (Figure 7) was carried out
using the following complementary Texas Red labelled
targets:

P5' complementary target to P5 sequence:

5'Texas Red-TCGGTGGTCGCCGTATCATT-3'

P7' complementary target to P7 sequence:

5'Texas Red-TCGTATGCCGTCTTCTGCTTG-3'

The target volume was 0.1 ml and the concentration was 0.5
μM. The target was made up in two different hybridisation
buffers and tested. The composition of the TAQ PCR buffer
was 1M betaine, 1.3% DMSO, 10 mM Tris, 1.5 mM MgCl2 and 50
mM KCl (pH 9). The other buffer contained 5XSSC (diluted
from a 20X stock) and 0.1% (v/v) Tween 20 (pH 7). A special
PCR film was used to seal the wells of the microplate and
prevent evaporation on heating. The plate was then placed on
a PCR block with lid and submitted to the following
conditions:

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1) 0.5 °/s to 97.5 °C

2) 97.5 °C for 2 mins 30 secs

3) 97.5 °C for 2 secs – 0.1 °C per cycle

4) Goto 3, 574 times

5   5) 40.0 °C for 15 mins

6) End

After heating and cooling the plate was washed 15 times
using the microplate washer (modified program 'AHPEM170')
10   with 5XSSC, 0.1% v/v Tween 20 pH 7, then 6 times using the
same program but with 0.1XSSC, 0.1% v/v Tween 20 pH 7.
Following washing the plate was scanned wet under on a
Typhoon 9600 imager at 700V with the ROX filter, 633 nm
excitation, 200 µm pixel size.

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Results:

The results presented in Figure 8 show a clear improvement
in hybridisation signal for both the P5 and P7 primer upon
adding a spacer with 10 T bases. Not only is the signal much
20   higher but the signal-to-noise (specific to non-specific
hybridisation) also improves immensely.

(2)   Hybridization on Typhoon slides – effect of spacer

25   **Coupling of oligo primers to SFA surface:**

The following 4 primers were coupled to the surface at 1.0
µM concentrations from 10 mM phosphate buffer pH 7:

1) P7 primer without polyT spacer:

5'phosphorothioate-CAAGCAGAAGACGGCATACGA -3'

2) P7 primer with polyT spacer:

5'phosphorothioate-TTTTTTTTTTCAAGCAGAAGACGGCATACGA -3'

3) P5 primer without polyT spacer:

5   5'phosphorothioate-AATGATACGGCGACCACCGA -3'

4) P5 primer with polyT spacer:

5'phosphorothioate-TTTTTTTTTTAATGATACGGCGACCACCGA -3'

Coupling was carried out for 1 hour by spotting 7 µl of each
10 oligo into one or more wells created by sticking a Grace
Biolab CultureWell coverglass gasket (gasket 1, Figure 9)
onto a Typhoon slide previously modified with 2% acrylamide,
2 mol% BRAPA silane-free acrylamide. During coupling the
slides were kept in the dark in a humidity chamber. After
15 coupling the slides were rinsed with 250 ml of 0.1 M
phosphate buffer pH 7 from a wash bottle. Then each slide
was vortexed 20 seconds in 10mM/10mM Tris/EDTA pH 8 buffer,
then rinsed with MilliQ water and dried.

20 **Hybridisation:**

Hybridisation to the primers (Figure 7) was carried out
using the following complementary Texas Red labelled
targets:

P5' complementary target to P5 sequence:

25 5'Texas Red-TCGGTGGTCGCCGTATCATT-3'

P7' complementary target to P7 sequence:

5'Texas Red-TCGTATGCCGTCTTCTGCTTG-3'

A silicone gasket (gasket 2, Figure 9) was attached to the
primer modified slide and a clean glass slide placed on top
to create a sealed chamber. Clips were used to ensure
sealing. The space created by the gasket was then filled
5   with one of the complementary targets (P5' or P7') then the
primer-modified side was placed in contact with an aluminium
heating block. A box was placed on top to prevent access of
light. The temperature of the aluminium block was increased
from room temperature to 95 °C (required 15 minutes). The
10 temperature of the heater was then turned down to 40 °C and
the slides allowed to cool to 50 °C (required 2 hours). Once
at 50 °C the slide was allowed to cool to room temperature
during 10 minutes. The complementary target solution was
then removed with a syringe and the inside washed by
15 injection 6X with 5XSSC, 0.1 % Tween 20. The gasket, clips
and slides were then dismantled and the primer-modified
slide rinsed with agitation in a tube containing 5XSSC, 0.1
% Tween 20 for 2 minutes. This was repeated then the primer-
modified slide was rinsed with 0.1XSSC, 0.1 % Tween 20,
20 twice for 2 minutes with agitation. The slide was then dried
with Argon and scanned dry at 700V as above.

Results:

The results presented in Figures 10 and 11 show a clear
25 improvement in hybridisation signal for both the P5 and P7
primer upon adding a spacer with 10 T bases. Not only is the
signal much higher but the signal-to-noise (specific to non-
specific hybridisation) also improves immensely.

30 **(3) Hybridization on Typhoon slides - effect of spacer
length**

Coupling of oligo primers to SFA surface:

The following 8 primers were coupled to the surface at 1.0 µM concentrations from 10 mM phosphate buffer pH 7:

5   1) P7 primer without polyT spacer:

5'phosphorothioate-CAAGCAGAAGACGGCATACGA -3'

2) P7 primer with polyT spacer:

5'phosphorothioate-TTTTTTTTTTCAAGCAGAAGACGGCATACGA -3'

3) P5 primer without polyT spacer:

10   5'phosphorothioate-AATGATACGGCGACCACCGA -3'

4) P5 primer with polyT spacer:

5'phosphorothioate-TTTTTTTTTTAATGATACGGCGACCACCGA -3'

5) P5 primer with polyT spacer:

5'phosphorothioate-TTAATGATACGGCGACCACCGA -3'

15   6) P5 primer with polyT spacer:

5'phosphorothioate-TTTTTAATGATACGGCGACCACCGA -3'

7) P5 primer with polyT spacer:

5'phosphorothioate-TTTTTTTTTTTTTTTTTTTTAATGATACGGCGACCACCGA -3'

20   8) P5 primer with C18 spacer:

5'phosphorothioate-C_{18}-AATGATACGGCGACCACCGA -3'

Coupling was carried out for 1 hour by spotting 7 µl of each oligonucleotide into one or more wells created by sticking a
25   Grace Biolab CultureWell coverglass gasket (gasket 1, scheme 2) onto a Typhoon slide previously modified with 2% acrylamide, 2 mol% BRAPA silane-free acrylamide. During coupling the slides were kept in the dark in a humidity

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chamber. After coupling the slides were rinsed with 250 ml
of 0.1 M phosphate buffer pH 7 from a wash bottle. Then each
slide was vortexed 20 seconds in 10mM/10mM Tris/EDTA pH 8
buffer, then rinsed with MilliQ water and dried.

5

Hybridisation:

Hybridisation to the primers (Figure 7) was carried out
using the following complementary Texas Red labelled
targets:

10   P5' complementary target to P5 sequence:

5'Texas Red-TCGGTGGTCGCCGTATCATT-3'

P7' complementary target to P7 sequence:

5'Texas Red-TCGTATGCCGTCTTCTGCTTG-3'

15   Hybridisation was carried out as above. A silicone gasket
(gasket 2, Figure 9) was attached to the primer modified
slide and a clean glass slide placed on top to create a
sealed chamber. Clips were used to ensure sealing. The space
created by the gasket was then filled with one of the
20   complementary targets (P5' or P7') then the primer-modified
side was placed in contact with an aluminium heating block.
A box was placed on top to prevent access of light. The
temperature of the aluminium block was increased from room
temperature to 95 °C (required 15 minutes). The temperature
25   of the heater was then turned down to 40 °C and the slides
allowed to cool to 50 °C (required 2 hours). Once at 50 °C
the slide was allowed to cool to room temperature during 10
minutes. The complementary target solution was then removed
with a syringe and the inside washed by injection 6X with
30   5XSSC, 0.1 % Tween 20. The gasket, clips and slides were
then dismantled and the primer-modified slide rinsed with

agitation in a tube containing 5XSSC, 0.1 % Tween 20 for 2
minutes. This was repeated then the primer-modified slide
was rinsed with 0.1XSSC, 0.1 % Tween 20, twice for 2 minutes
with agitation. The slide was then dried with Argon and
5   scanned dry at 700V as above.

Results:

The results presented in Figure 12 show that hybridisation
yield increases sharply with polyT spacer length up to 10Ts
10 but between 10T and 20T bases it begins to decrease. A
reduced hybridisation yield above an optimum spacer length
has also been reported elsewhere (Shchepinov, M.S. *et al.*
(1997) Nucl. Acids Res. 25, 1155-1161; Guo,Z. *et al* (1994)
Nucl. Acids Res. 22, 5456-5465). One possible explanation
15 could be that larger primers have lower primer density on
the surface and therefore result in a smaller hybridisation
signal. A C18 spacer results in only a small improvement in
hybridisation yield suggesting that spacer hydrophilicity is
also an important factor.

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All patents, patent applications, and published references
cited herein are hereby incorporated by reference in their
entirety. While this invention has been particularly shown
and described with references to preferred embodiments, it
25 will be understood by those skilled in the art that various
changes in form and details may be made without departing
from the scope of the invention encompassed by the claims.